UNIVERSITÀ DEGLI STUDI DI MILANO

Dottorato di ricerca in Medicina Clinica e Sperimentale

Ciclo XXIX

PHENOTYPIC AND FUNCTIONAL CHARACTERIZATION OF HUMAN NEUTROPHIL SUBSETS IN PATIENTS WITH COLORECTAL CANCER AND INFLAMMATORY BOWEL DISEASES

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INDICE

LIST OF ABBREVIATIONS

5’-ASA: mesalazine

6-MP: 6-mercaptopurine

ADA: adalimumab

AIEC: Adhesive-invasive E. coli

AZA: azathioprine

BSA: bovine serum albumin

CD: cluster differentiation

CD: Crohn’s disease

CRC: Colorectal cancer

CXCR4: CXC-chemokine receptor 4

DTT: dithiothreitol

EDTA: ethylenediamine tetraacetic acid

ELISA: Enzyme Immunosorbent Assay

ETC: transport chain

FBS: Fetal Bovine Serum

GALT: gut-associated lymphoid tissue

G-MDSC: granulocytic human myeloid-derived suppressor cells

GWAS: genome wide association study

HBSS: Hank's balanced salt solution

IBD: Inflammatory bowel diseases

IFN-y: interferon gamma

LPS: lipopolysaccharide

MAP: Mycobacterium avium spp. paratuberculosis

MCSF: macrophage colony stimulating factor

MDSC: myeloid derived suppressor cells

MFI: Mean Fluorescence Intensity

MMP9: matrix metalloproteinase 9

MPO: myeloperoxidase

NETs: neutrophil extracellular traps

NLR: neutrophil-to-lymphocyte ratio

PMA: phorbol myristate acetate

PMN: polymorphonuclear leukocytes

RBC: red blood cells

ROS: reactive oxygen species

SAA-1: serum amyloid A-1

TAM: tumor-associated macrophages

TAN: tumor-associated neutrophils

TGFβ: transforming growth factor-β

TLRs: toll-like receptors

TMB: 3,3′,5,5′-Tetramethylbenzidine

UC: ulcerative colitis

VEGF: vascular endothelial growth factor

CHAPTER 1

INTRODUCTION

1. 1 NEUTROPHILS

1.1.1 Origin and main characteristics

Neutrophils (also called polymorphonuclear leukocytes, PMN) are the most abundant white blood cells in humans (1). Humans and mice differ in the number of circulating neutrophils: in humans, 50–70% of circulating leukocytes are neutrophils, as compared to only a 10–25% in mice (2, 3). PMN are generated in the bone marrow at a rate of 10^{11} cells/day (4). In the circulation, mature neutrophils have an average diameter of 7–10 μm, their nucleus is segmented and their cytoplasm is enriched with granules and secretory vesicles (5).

PMNs were always considered short-lived cells with a half-life in the circulation of approximately 1.5 and 8 hours in mice and humans, respectively (6, 7). Nevertheless, a recent study challenged this concept, proposing that under basal conditions the average circulatory lifespan of neutrophils might rise up to 12.5 hours for mouse cells and 5.4 days for human neutrophils (6).

During inflammation, neutrophils become activated by cytokines, growth factors, and bacterial products (8, 9) and their longevity increases by several fold. This process ensures a longer presence of primed neutrophils at the site of inflammation (8,10). A longer lifespan may allow neutrophils to carry out more complex activities in a tissue, such as contributing to the resolution of inflammation or shaping adaptive immune responses, but their persistence in tissues may lead to bystander cell injury.

Data from experimental and clinical settings show that the re-localization of neutrophils to the site of inflammation is crucial for clearance of infections, although an excessive infiltration and activation of neutrophils at the site of tissue damage can

cause chronic inflammation, limit injury repair and lead to loss of organ function (11,12). Indeed, a marked decrease in neutrophil numbers in the blood leads to severe immunodeficiency in humans (13,14). Thus, the recruitment and the functions of neutrophils are tightly controlled.

In most tissues, the leukocyte recruitment cascade involves the following commonly recognized steps: tethering, rolling, adhesion, crawling and, finally, transmigration, as shown in Figure 1. Neutrophil recruitment is initiated by changes on the surface of endothelium, as a result of stimulation by inflammatory mediators (including histamine and cytokines) released by tissue-resident sentinel leukocytes upon contact with pathogens (15-17). Once neutrophils are recruited, specific molecule expression is modulated in order to ensure rolling (selectins) adhesion and migration (integrins) through the endothelium.

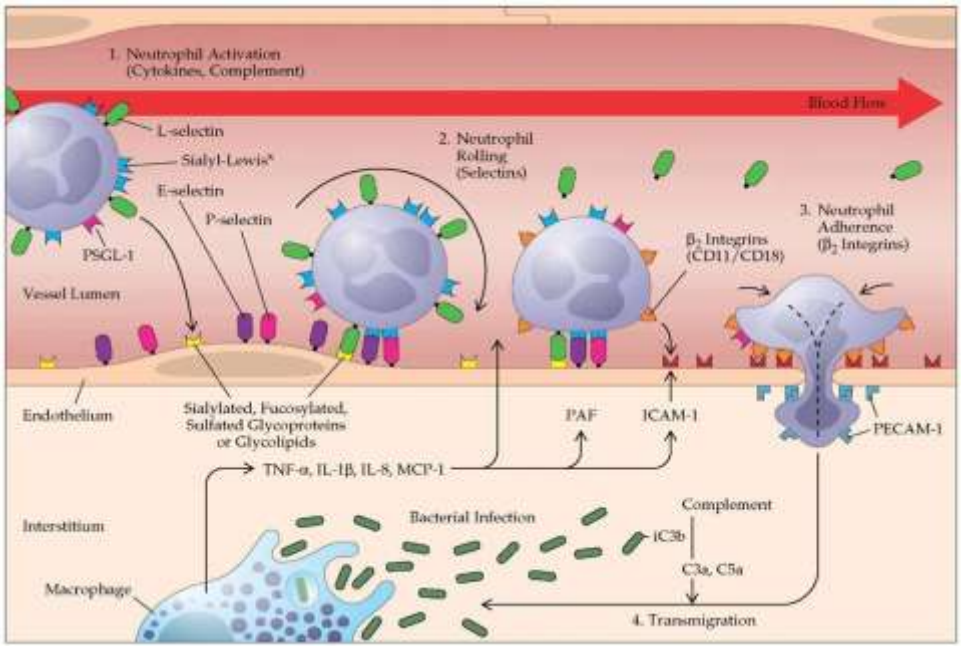

Fig.1 Leukocyte extravasation. This process concerns the movement of leukocytes out of the circulatory system and towards the site of tissue damage or infection. The different steps of the adhesion cascade are capture, rolling, slow rolling, firm adhesion, and transmigration. Each of these steps appears to be necessary for effective leukocyte recruitment (W. A. Muller, 2013).

1.1.2 Neutrophil functional roles

Neutrophils play a well-established role in host defense against pathogens (18, 19). PMNs detect the presence of pathogens through germ line-encoded receptors that recognize microbe-associated molecular patterns. In vertebrates, the most characterized receptors are Toll-like receptors (TLRs). Ten human TLRs have been identified which enable responses to a range of pathogen-associated molecules including LPS (TLR4), lipoproteins and peptidoglycans (TLR2 in combination with TLR1 or TLR6), flagellin (TLR5), double-stranded (viral) RNA (TLR3), and bacterial DNA (CpG motifs, TLR9). In addition, TLR4 recognizes not only LPS but also non-infective inflammatory stimuli such as heat shock protein 60 (HSP60) and fibrinogen peptides (20-23).

An increasing number of studies have documented that TLR-induced cytokine expression by neutrophils can be influenced, either positively or negatively, by immunomodulating factors such as IFNγ (24, 25) and IL-10 (26), respectively.

Recently it has been shown that melanoma patients possess a higher number of circulating neutrophils that secrete IL-10 after recognition of serum amyloid A-1 (SAA-1), one of the first and most abundant proteins secreted during the physiological response to infections and injuries. In this work it was suggested that this process, together with the functional interaction with immune cell subsets such as iNKT cells, might directly control the plasticity of neutrophil differentiation (27).

Once detected, PMNs target microorganisms through a number of processes including (Figure 2):

i) degranulation through the release of granular antimicrobial peptides such as myeloperoxidase (MPO), neutrophil elastase and matrix metalloproteinases (MMPs);

ii) phagocytosis and degradation via synthesis of reactive oxygen species (ROS) inside phagolysosomes

iii) microbial trapping by extrusion of a meshwork of chromatin bound to granular peptides termed neutrophil extracellular traps (NETs) (28).

Neutrophils contain in their cytoplasm granules necessary to perform their functions. PMN granules have been classified into three types: azurophilic (primary) granules, specific (secondary) granules and gelatinase (tertiary) granules (29). Azurophilic granules can be distinguished from the other types by their uptake of basic dye azure A, owing to their acid mucopolysaccharide content, and contain myeloperoxidase (MPO), azurocidin, bacterial permeability-increasing protein, cathepsin G, defensins, elastase and human neutrophil peptides. Secondary granules contain cathelicidin antibacterial peptide (also known as antibacterial peptide LL-37), lactoferrin and neutrophil gelatinase-associated lipocalin. Tertiary granules contain peptidoglycan recognition proteins. Lysozyme is present in all three types of granules. Together these antimicrobial granular proteins have an important role in the activation of innate and adaptive immunity (30).

Neutrophils are also able to synthetize reactive oxygen species (ROS), that is a collective term that describes the chemical species that are formed upon incomplete reduction of oxygen and includes the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and and hypochlorous acid (HOCl) (31).

ROS are generated as by-products of cellular metabolism through the electron transport chain (ETC) in mitochondria as well as via the cytochrome P450. The other major source in which ROS are not produced as by-products, are the NADPH oxidases that are present in a variety of cells, especially the professional phagocytes and endothelial cells (32), which are central to the genesis of the inflammatory response (33).

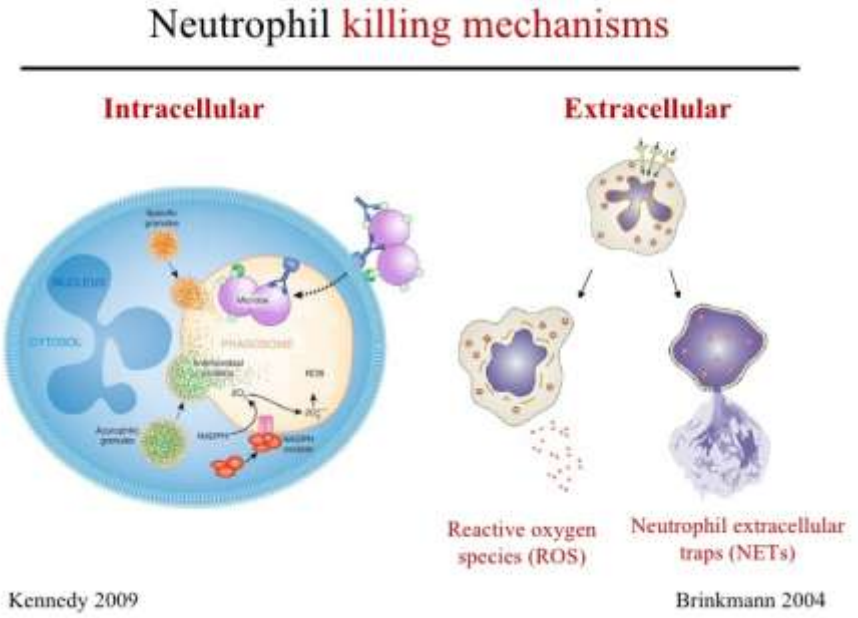

Fig. 2 Neutrophils killing mechanisms. Neutrophils kill pathogens through intracellular mechanism such as phagocytosis, and extracellular mechanisms such as ROS production and NETosis (Segal A., 2005; Zawrotniak M., 2003).

In 2004, the group of Brinkmann and Zychlinsky described how after stimulation with interleukin-8 (IL-8), phorbol myristate acetate (PMA), or lipopolysaccharide (LPS), neutrophils formed previously unknown NET-like structures called neutrophil extracellular traps (NETs) (34).

These structures were composed by nuclear chromatin, associated mainly with nuclear histones and many granular antimicrobial proteins, and with cytoplasmic proteins (35). Moreover, NETs are also generated and released during a distinct process of cell death called NETosis (Fig.3) (36). In addition to triggering innate immune responses, neutrophils regulate the adaptive immune response (37) and NETosis also contributes to the activation of the immune system during inflammation (38, 39). Neutrophils can interact with B cells (40, 41), T cells (42, 43) and antigen presenting cells in lymphoid organs (44, 45). The cytokines, proteases and other factors contained in PMN cytoplasmic granules and released during PMNs functional

activation, can act by directly regulating the activity of the adaptive immune response, including both B cell and T cell activation. The type of response, the cytokine milieu and the cell types encountered during the course of the immune activation contribute to shaping the phenotype and function of neutrophils, similarly to what is observed for macrophages (46-50).

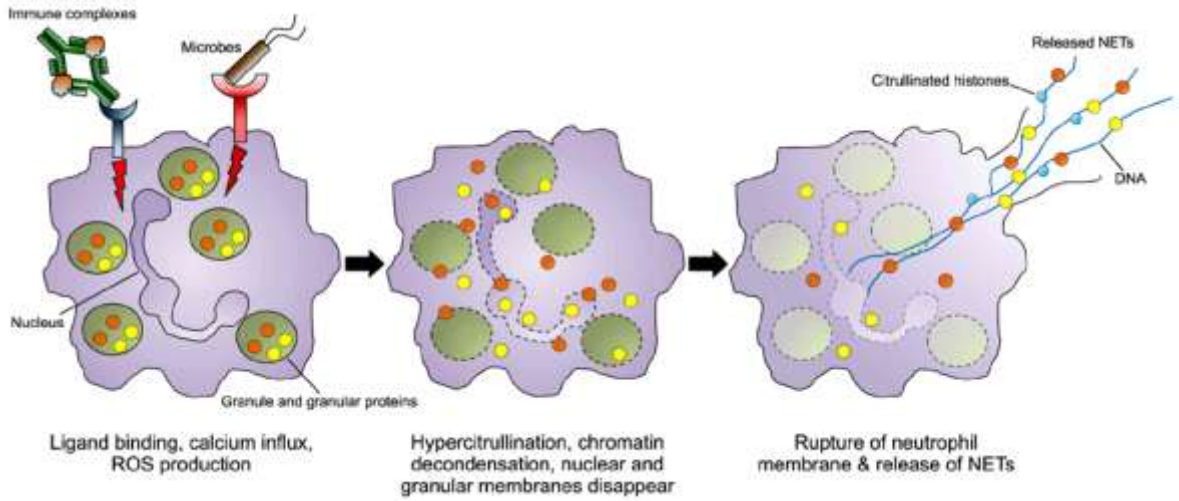

Fig.3 NETs Formation. Neutrophils kill pathogens through the formation of extracellular fibers named NETs, primarily composed of proteins from azurophilic granules (neutrophil elastase, cathepsin G and myeloperoxidase) and DNA of the same cells, which bind pathogens (Zawrotniak M., 2003)

1.2 Inflammatory Bowel Diseases

1.2.1 Epidemiology

Inflammatory bowel diseases (IBD) are a chronic, relapsing, inflammatory disorders of the gastrointestinal tract and include ulcerative colitis (UC) and Crohn's disease (CD), which differ in the pathology and clinical characteristics. These pathologies are characterized by abdominal pain, fever, chronic diarrhea, and rectal bleeding due to ulceration of the inner lining of the colon and/or rectum, which can be accompanied by complications such as fistulation, stenosis, and abscesses in CD and megacolon in UC. Currently, the etiology and pathogenesis of IBD are still poorly understood. It is widely accepted that IBD arise in genetically predisposed individuals as the result of an exaggerated reaction of gut-associated lymphoid tissue (GALT) to bacterially-derived antigens (51). IBD arise in young adults and manifest a chronic course. The incidence of IBD varies according to geographic location, but in the last decade it has been observed a general increase worldwide. Since the middle of the twentieth century, the incidence of ulcerative colitis and Crohn's disease has increased in the Western world, including North America, Europe, Australia and New Zealand. Currently, the prevalence of IBD in the Western world is up to 0.5% of the general population (52) (Fig.4).

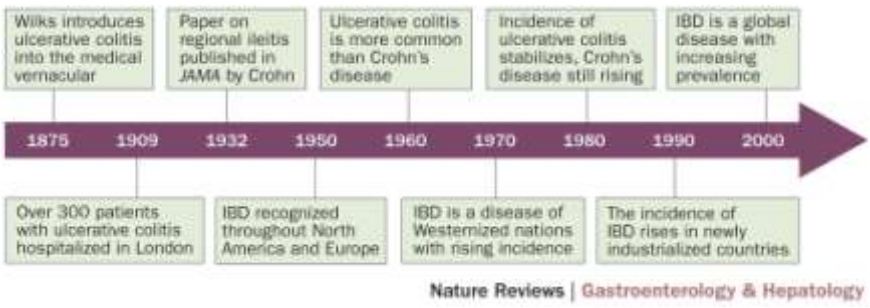

Fig. 4. Historical timelines of CD and UC diseases throughout the world

Over the past few decades, newly industrialized countries in Asia, South America and the Middle East have documented the emergence of IBD (53-58). The incidence of IBD in these newly industrialized countries is still considerably lower than that in the Western world, but IBD is considered a global disease (59), as shown in Figure 5.

Fig. 5. The global prevalence of IBD in 2015

A variety of environmental and behavioural factors are linked to IBD pathogenesis, including diet, smoking, stress, sleep patterns, hygiene, and antibiotic use (60). Considerable advances have been made in recent years in the understanding of the role of the genes, the intestinal immune system, and the gut microbiome in the pathogenesis of CD and UC (61, 62).

1.2.2 Genetics in IBD

The association of IBD susceptibility genes with bacteria has recently been highlighted. Epidemiological studies of IBD have revealed its polygenic and multifactorial nature (63, 64). Genetic analyses of IBD patients have identified over 200 loci associated with UC or CD risk (65-67).

Interestingly, several of the identified risk loci play also significant roles in determining susceptibility to infections and to the host-microbial response, further strengthening the interaction between the gut microbiome and the intestinal immune system in the development of IBD. Risk genes included *nod2* in CD (69, 70), *il-10*, *il23r*, and *atg16l1* (71) (Fig.6).

A recent meta-analysis of GWAS reported 163 genetic loci associated with IBD (72). Of these, 110 loci were shared by UC and CD, while 30 and 23 loci were specific to CD and UC, respectively (73). However, candidate genes that in patients functionally contribute to disease susceptibility have not been confirmed by GWAS, underlying the complexity of these diseases.

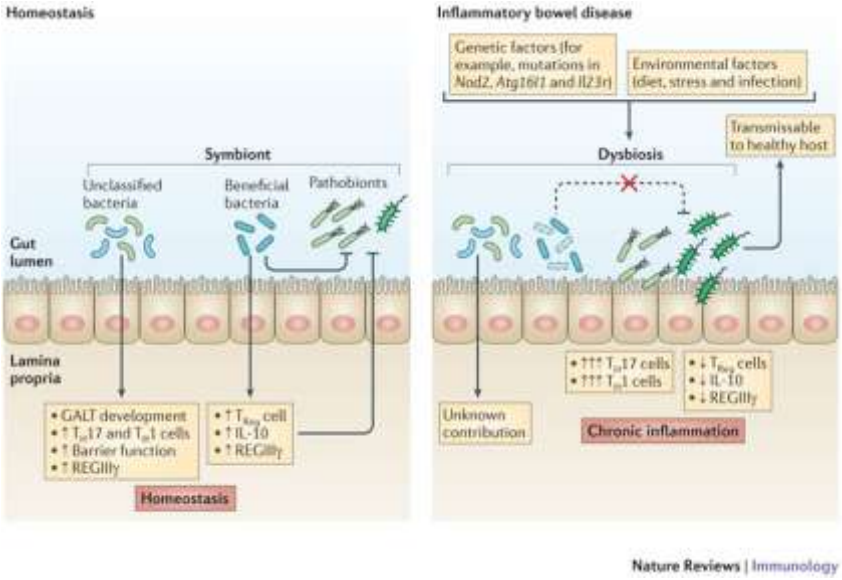

Fig.6 During homeostasis beneficial subsets of commensal bacteria tend to have anti-inflammatory activities. In inflammatory bowel disease (IBD) a combination of genetic factors (for example, mutations in nucleotide-binding oligomerization domain 2 (Nod2), autophagy-related gene 16-like 1 (Atg16l1) and interleukin-23 receptor (Il23r) and environmental factors (such as infection, stress and diet) result in dysbiosis (Ballester V., 2004).

1.2.3 The role of the gut microbiota in IBD

The gut is a complex environment composed of immune cells, epithelial cells, stromal cells and the intestinal microflora. The host provides the gut bacteria with a nutrient-rich environment and bacteria, in turn, contribute to the host homeostasis through short-chain fatty acids and essential vitamins production. This mutual relationship between the host and the gut bacteria is called symbiosis. Recent advancement of next-generation sequencing techniques has enabled culture-independent analysis of the gut microbiota, revealing that an altered balance of the gut microbiota constituents, rather than specific pathogens, is involved in the pathophysiology of several diseases. This shift in the balance of the gut microbiota composition is referred to as dysbiosis. The normal gut microbiome comprises 100 trillion diverse microbes, mostly bacteria, with at least 160 species in each individual (74).

More than 90% of the human gut microbiota is composed of four major phyla. The Firmicutes (49-76%) and Bacteroidetes (16–23%) phyla dominate, followed to a much less extent by the Proteobacteria and Actinobacteria phyla (75, 76). The Firmicutes phylum is mainly composed by the Clostridium XIV and IV groups.

IBD patients show a marked dysbiosis as compared to healthy individuals. The most well defined change that has been noted in patients with IBD is the reduced abundance of the phyla Firmicutes (77-79). Amongst the Firmicutes, the reduced presence of *Faecalibacterium prausnitzii* has been well documented in patients with CD as opposed to controls (80-83). Additionally, it has been suggested that there may be spatial reorganization of the Bacteroides species in patients with IBD, with *Bacteroides fragilis* being responsible for a greater proportion of the biofilm mass in patients with IBD compared to controls, due to increased adherence (84).

Most of the known pathogenic bacteria in humans belong to the phylum Proteobacteria, which have been increasingly found to have a key role in IBD (85). Increased concentrations of Escherichia coli including pathogenic variants have been documented in ileal CD (86, 87).

This interesting shift within the gut microbiome with a decrease in obligate anaerobes of the phylum Firmicutes and an increase in facultative anaerobes of Proteobacteria has given rise to a putative "oxygen" hypothesis wherein disruption in anaerobiosis points to a role for oxygen in intestinal dysbiosis (88). The ileal CD patients were found to have alterations in bacterial carbohydrate metabolism, bacterial-host interactions, as well as human host-secreted enzymes (89).

There are several specific bacteria that are associated with IBD. *Mycobacterium avium spp. paratuberculosis* (MAP) causes chronic granulomatous ileitis (Johne's disease) in cattle and sheep, which shares some pathological features with CD (90). Adhesive-invasive *E. coli* (AIEC), which can adhere to and invade the intestinal epithelial cells, colonize the ileal mucosa of CD (91). AIEC also replicate in macrophages and stimulate TNFα production from macrophages. It was observed that *Fusobacterium varium* attaches to inflamed regions in UC and invades the mucosa at ulcers (92). *F. varium* produces butyrate, and rectal administration of butyrate has been shown to cause mucosal damage in mice (93).

1.2.4 Neutrophils and IBD

In IBD patients it is observed a robust inflammatory response associated with mucosal injury, increased epithelial permeability and invasion of commensal bacteria into the subepithelial space or lamina propria (94). Circulating neutrophils sense the chemoattractant gradient originated by the inflammatory responses in the gut and traverse the vascular endothelium to reach the intestinal lamina propria. Nevertheless, once recruited to the lamina propria, the relative contribution of PMN to the pathogenesis of IBD is controversial. Some studies describe a beneficial role by neutrophils in resolving intestinal inflammation, yet others report pathological contribution.

During intestinal inflammation, neutrophils secrete significant amounts of pro-inflammatory cytokines such as CXCL8 and IL-17 (95) in response to IL-23 and IL-6 (96), which has been widely implicated in pathological intestinal inflammation (97). Another potential contribution of PMN to pathological immune cell activation includes the ability to acquire antigen-presenting function during colitis, an event previously reported also during rheumatoid arthritis (98). PMN isolated from inflamed colon in a murine model of adoptive transfer of CD4+ T cells were reported to express major histocompatibility complex-II and CD86, usually present on antigen-presenting cells such as macrophages or dendritic cells but not on PMN. Such neutrophils were capable of inducing in vitro CD4+ T cell activation in a major histocompatibility complex-II- and antigen-dependent manner (99). Although these findings have not been confirmed in other murine colitis models or in human disease, it is intriguing that PMN might contribute to the pathogenesis of colitis by activating T cells.

Finally, during the inflammatory response, neutrophils are capable to secrete anti-inflammatory cytokines, including interleukin (IL)-10 (100-102). IL-10 down-regulates major histocompatibility complex-II (MHC class II) expression (103) and the expression of the co-stimulatory ligands CD80/CD86 (B7-1, B7-2) in monocytes, macrophages and dendritic cells (104). Moreover PMNs contribute to the recruitment of immune cells that facilitate mucosal healing by secreting chemokines and by releasing mediators necessary for the resolution of inflammation (105).

1.3 Colorectal Cancer (CRC)

1.3.1 Epidemiology and Risk Factors

Colorectal cancer (CRC) is an important public health problem with nearly one million new cases diagnosed world-wide each year and half a million deaths. It accounts for over 9% of all cancer incidence and it is the third most common cancer worldwide and the fourth most common cause of death. CRC affects men and women almost equally with a slight male predominance for rectal cancer (108), and the highest incidence rates occur in Australia, New Zealand, Canada, the United States, and parts of Europe. The countries with the lowest risk include China, India, and parts of Africa and South America (106, 107).

Several risk factors are associated with the incidence of colorectal cancer. Those that an individual cannot control include age and hereditary factors. In addition, a substantial number of environmental and lifestyle risk factors may play an important role in the development of colorectal cancer, that include physical inactivity, obesity, high consumption of red processed meat, smoking, and moderate-to-heavy alcohol consumption (109).

1.3.2 IBD- associated colorectal cancer

Patients with inflammatory bowel diseases (IBD) have an increased risk of developing colorectal cancer, even though no genetic correlation has been identified yet. The mortality rate of patients suffering from IBD who have been diagnosed with colorectal cancer is higher than that occurring for sporadic colorectal cancer. The risk of developing colorectal cancer in patients with IBD has been reported to be between 7% and 14% in multiple studies after a disease duration of 25 years (110, 111). Some studies have reported the risk to be as high as 30% in patients who have had IBD for greater than 35 years (112, 113).

1.3.3 Bacteria and Cancer

Several human studies have demonstrated a link between the gut microbiota composition and CRC development. Initial studies associated abundance of Bacteroides and Bifidobacteria with increased risk of developing colon polyps, whereas Lactobacillus and *Eubacterium aerofaciens* presence were shown to be protective (116). Other studies also reported an association between the abundance of *Escherichia Coli* and Colorectal Adenomas and Cancer development (117) and between an higher abundance of hydrogen sulfide (H_2S) producing bacteria and increased risk of colon cancer (118). More recent studies that took advantage of high-throughput molecular sequencing methods, revealed changes in fecal microbiota composition in CRC subjects as compared to healthy individuals (119, 120). Patients with colorectal adenomas showed a concomitant reduced abundance of Clostridia, Roseburia, Eubacteria spp., and other butyrate-producing bacteria in fecal samples of adenoma subjects compared with healthy controls (121). Lactobacillus and Bifidobacterium, benefit the host through anti-inflammatory and anti-tumorigenic properties, as well as through pathogen exclusion (122-124). It was also demonstrated an overabundance of *Fusobacterium Nucleatum* within the tumor site compared with

normal tissue (125). Other specific strains of bacteria have been classified as cancerogenic pathogens, such as *Streptococcus bovis*, *H. pylori*, and *Enterococcus faecalis* (126,176,177).

Most of the disease-promoting and pro-carcinogenic effects of these pathobionts depend on virulence factors (127), in particular on *afa* and *eae* adhesins for E.coli (128, 129) *FadA* virulence factor for *F. nucleatum* (130) or, *CagA* or *VacA* for *H. pylori* (131, 132); *B. fragilis* can also exert a virulent potential through the production of the metalloprotease toxin BFT. The so called enterotoxigenic *B. fragilis*, that comprises a relatively small proportion of fecal microbiota (approximately 0.5%-1%), is more prevalent in late-stage CRC, suggesting a possible role of BFT in CRC promotion and progression (133) (Fig.7)

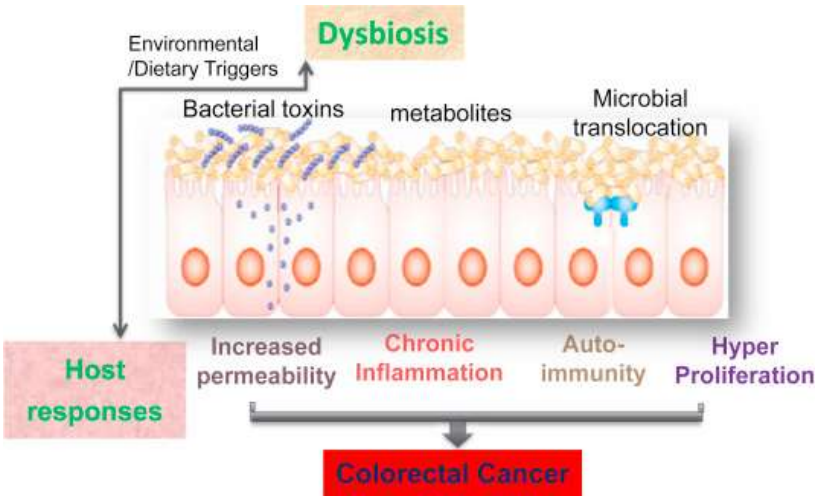

Fig. 7. Possible mechanisms by which the gut bacterial microbiota participates in colorectal carcinogenesis (Gagnière J, 2016).

1.3.4 Neutrophils and Cancer

In recent years, accumulating data suggest important and significant roles for neutrophils in tumor biology (134, 135), given that they have been found in vivo in close association with tumor cells and within tumour vasculature (136). However, the exact role of neutrophils in the tumor microenvironment is controversial. Many patients with advanced cancer show elevated blood neutrophilia (137), which has been associated with poorer prognosis in many types of cancers, including bronchoalveolar carcinoma (138), metastatic melanoma (137) and renal carcinoma (139). On the contrary, the neutrophil- to- lymphocyte ratio has been introduced as a prognostic factor in many tumor types, including colorectal cancer and non-small cell lung cancer (140). An elevated neutrophil-to-lymphocyte ratio (NLR) of the peripheral blood, reflecting the systemic immune response, has been correlated with poor clinical outcome in patients with advanced CRC (141). CRC patients with elevated NLR (>5) have tumors characterised by aggressive biology and a distinctive expression profile of cytokines involved in angiogenesis, inflammation, and regulation of the EGF axis (142). Neutrophils can make up a significant portion of the inflammatory cell infiltrate in many models of cancer, and it has been shown that tumor cells themselves mediate neutrophil recruitment to the tumour (143).

When neutrophils traffic into tumors, they are referred to as Tumor-associated neutrophils (TAN).

1.3.5 Tumor infiltrating-cells: MDSC, TAMs and TANs

The tumor microenvironment includes tumor cells, the surrounding blood vessels, the extracellular matrix, the immune infiltrate and, in intestinal tumors, also commensal bacteria. Among infiltrating immune cells, an important role is played by myeloid derived suppressor cells (MDSC), a heterogeneous group of myeloid cells characterized by potent immune-suppressive activity. In mice, they are generally characterized as Gr-1^{+}CD11b^{+} cells. Under physiological conditions, immature myeloid cells with this phenotype are present in bone marrow and in the spleen, and lack immune-suppressive activity. In contrast, in a tumor-bearing host, there is a dramatic expansion of cells with the same phenotype and immune-suppressive activity in various tissues (144).

In recent years, in tumor-bearing mice, two major groups of cells with phenotypic and functional characteristics of MDSCs were identified: cells with morphology and phenotype typical for monocytes were called M-MDSC (CD11b^{+}Ly6C^{high}Ly6G^{-}), and cells with morphology and phenotype typical for granulocytes were named G-MDSC (CD11b^{+}Ly6C^{low}Ly6G^{+}) (145-147). In humans, MDSC are typically defined as CD11b^{+}CD33^{+}CD34^{+} CD14^{-}HLA-DR^{-}, with a variable expression of CD15 (148). It has been previously proposed that MDSC entering the tumor microenvironment can differentiate into tumor-associated macrophages (TAM) or tumor-associated neutrophils (TAN) in mice, and can promote tumor growth by favouring angiogenesis, hypoxia and immunosuppression (149, 150); in humans the relationship between TAMs, TANs and MDSC has not been fully clarified.

TAMs were firstly described in the early 1980s (151, 152). Analogously to the old T helper cell dichotomy, Th1 and Th2, macrophages exist in two distinct polarized states: one is the classically activated state (M1) and the other is the alternatively activated state. (M2). M1 macrophages possess antitumor activity, whereas M2 macrophages promote tumor invasion and metastasis (153). The M1 activation is

induced by intracellular pathogens, bacterial cell wall components, lipoproteins, and cytokines such as interferon gamma (IFN-y) and tumor necrosis factor alpha (TNF-α). Activated M1 macrophages secrete inflammatory cytokine and produce nitric oxide (NO), resulting in an effective pathogen killing function (154).

The M2 activation is induced by fungal cells, parasites, immune complexes, complements, apoptotic cells, macrophage colony stimulating factor (MCSF), interleukin-4 (IL-4), IL-13, IL-10 and tumor growth factor beta (TGF-β) (155). The M2 macrophages have high phagocytic capacity, produce extracellular matrix components, angiogenic and chemotactic factors, and IL-10 (156, 157).

Those TAMs demonstrating enhanced expression of CD163 (hemoglobin scavenger receptor), CD204 (class A macrophage scavenger receptor), CD206 (mannose receptor, C type 1), stabilin-1, arginase-1, and accelerated production of IL-10, VEGF, PGE2, and MMP9, generally show characteristics of M2 macrophages (158-160) (Fig. 8).

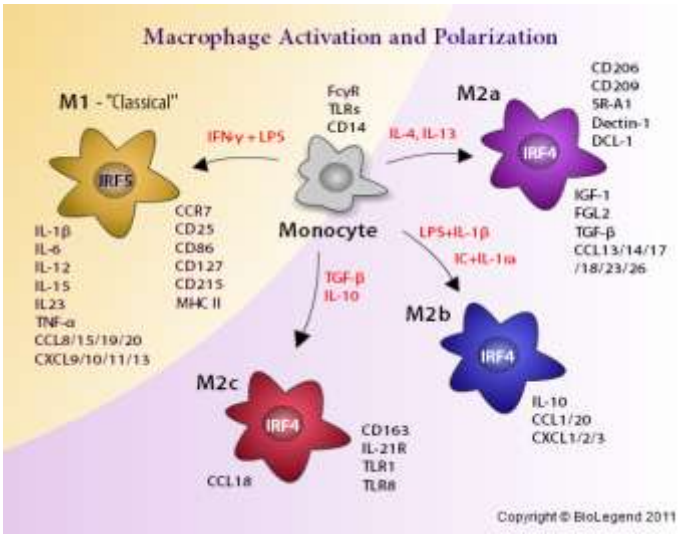

Fig. 8 Changes in macrophage phenotype. Plasticity of macrophage, when infiltrating tissue (Quatromoni JG. et al., 2012)

The critical functions of tumor-associated neutrophils (TANs) have only recently become appreciated. Surface markers used for the identification of neutrophils in tumor tissues include CD66b and CD15 (161). CD66b is GPI- anchored glycoprotein of the carcinoembryonic antigen (CEA) family and is located in the specific granules (162) and is associated to the aggregate formation of human neutrophils (163, 164).

Murine neutrophils that remained longer in the circulation were shown to express reduced levels of CD62L (also known as L-selectin), while expression of CXCR4 increased over time (165). In human lung tumor, compared with blood neutrophils, TANs displayed an activated phenotype ($CD62L^{low}CD54^{high}$) with a distinct repertoire of chemokine receptors that included CCR5, CCR7, CXCR3, and CXCR4.

Functionally, both TANs and neutrophils isolated from distant non-malignant lung tissue were able to stimulate T cell proliferation and IFN-γ release. Cross-talk between TANs and activated T cells led to substantial upregulation of CD54, CD86, OX40L, and 4-1BBL costimulatory molecules on the neutrophil surface. Recent studies have demonstrated also the expression of co-inhibitory molecules, such as PD-L1, in neutrophils following in vitro exposure to cytokines or after stimulation by LPS or other toll-like receptor ligands (TLRs) (166, 167).

PD-L1 expression on neutrophils was also increased in vivo in patients with active tuberculosis (TB) or HIV infection while it was decreased in patients who received anti-TB therapy or anti-viral treatment (168, 169). Subsets of neutrophils have also been defined by surface receptor expression and density (low-density neutrophils versus high- or normal-density neutrophils) (170).

For example, CXCR4 expression is increased in aged or senescent neutrophils and is associated with neutrophil trafficking to the bone marrow. Other changes in the expression of surface proteins on neutrophils have been described, including

intercellular adhesion molecule 1 (ICAM1) expression associated with systemic inflammation and reverse migration (171).

Similarly to TAM, also TANs appear to have dichotomous pro-tumorigenic and anti-tumorigenic effects (172, 173). Tumor-associated neutrophils can be designated as N1 (anti-tumorigenic and pro-inflammatory) and N2 (pro-tumorigenic and immunosuppressive). N2 neutrophils are characterized by high expression of CXC-chemokine receptor 4 (CXCR4), vascular endothelial growth factor (VEGF) and matrix metalloproteinase 9 (MMP9) and are induced in the presence of high levels of transforming growth factor-β (TGFβ). By contrast, N1 neutrophils expressing pro-inflammatory cytokines and chemokines, can kill cancer cells and are induced by inhibition of TGFβ signalling (Fig. 9) (174). An increase of IFN-β can also alter the phenotype of TANs to a more anti-tumorigenic phenotype (N1) (175).

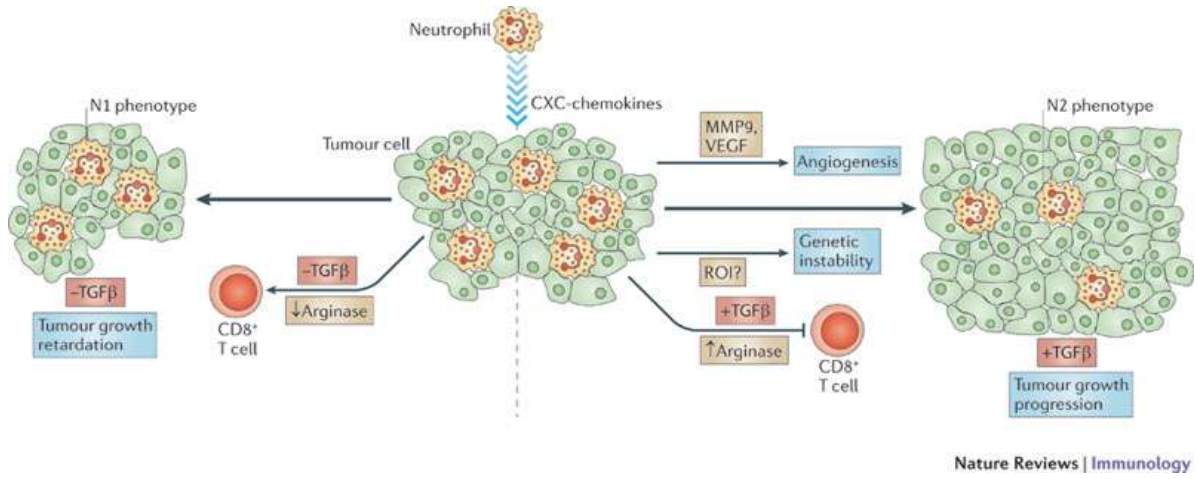

Fig. 9 Plasticity of neutrophils . Neutrophils are driven by transforming growth factor-β (TGFβ) to acquire a polarized, pro-tumoural N2 phenotype (characterized by high levels of arginase expression). By contrast, inhibition of TGFβ promotes a reprogramming of neutrophils to an N1 phenotype. This is associated with higher cytotoxic activity, higher capacity to generate hydrogen peroxide, higher expression of tumour necrosis factor (TNF) and intercellular adhesion molecule 1 (ICAM1), and lower expression of arginase (Perobelli SM et al., 2015).

CHAPTER 2

MATERIALS AND METHODS

2.1 PATIENTS

A total of 33 patients (17 with CD, 6 with UC and 10 with CRC) were enrolled in this study. IBD patients were classified according to disease, age, and concomitant therapies (mesalazine = 5'-ASA, azathioprine=AZA and steroids) that include biological drugs (Adalimumab=ADA). CRC patients were classified according to age, staging of tumor and concomitant therapies; as controls, a total of 16 patients (6 patients with diverticulitis and 10 non-CRC patients) were enrolled. The intestinal segments resected and clinical characteristics of the patients and controls are summarized in Table 1, Table 2, Table 3 and Table 4.

TABLE 1. Baseline characteristics of IBD patients

SUBJECTS	SEX	DISEASE	AGE	INTESTINAL SEGMENTS	THERAPY
1	**F**	CD	48	Colon	-
2	**M**	CD	41	Colon	-
3	**F**	CD	49	Colon	-
4	**F**	CD	47	Colon	-
5	**F**	CD	34	Ileum	-
6	**M**	CD	29	Colon	-
7	**F**	CD	76	Colon	-
8	**M**	CD	51	Colon	-
9	**F**	CD	45	Ileum	-
10	**F**	CD	52	Colon	-
11	**M**	CD	40	Colon	-
12	**M**	CD	35	Ileum	AZA
13	**F**	CD	47	Ileum	AZA
14	**F**	CD	50	Ileum	AZA
15	**F**	CD	38	Colon	5’-ASA
16	**M**	CD	57	Colon	5’-ASA
17	**F**	UC	18	Colon	-
18	**F**	UC	43	Colon	-
19	**M**	UC	48	Colon	-
20	**M**	UC	50	Colon	5'-ASA
21	**F**	UC	46	Colon	5'-ASA
22	**M**	UC	41	Colon	steroids
					BIOLOGICAL
23	**F**	CD	49	Colon	ADA

AZA= azathioprine; 5’-ASA= 5-Aminosalicylic acid (mesalazine); ADA=Adalimumab

TABLE 2. Baseline characteristics of CRC patients

SUBJECTS	DISEASE	AGE	TNM STAGING	THERAPY
1	CRC (IA)	84	I	-
2	CRC (IA)	69	I	-
3	CRC (IA)	82	I	-
4	CRC (IA)	74	IIa	steroids
5	CRC (IA)	71	IIa	steroids
6	CRC (IA)	81	IIa	steroids
7	CRC (IA)	76	IIa	steroids
8	CRC (IA)	62	IIIb	steroids
9	CRC (IA)	64	IIIb	-
10	CRC (IA)	67	IIIb	-

CRC= Colorectal cancer; IA= intra-tumoral area;

TABLE 3. Baseline characteristics of healthy controls (blood)

SUBJECTS	DISEASE	AGE	THERAPY
1	Diverticulitis	81	-
2	Diverticulitis	81	-
3	Diverticulitis	44	-
4	Diverticulitis	74	-
5	Diverticulitis	63	-
6	Diverticulitis	68	-

TABLE 4. Baseline characteristics of healthy controls (tissue)

SUBJECTS	DISEASE	AGE	THERAPY
1	Non-CRC	84	-
2	Non-CRC	69	-
3	Non-CRC	82	-
4	Non-CRC	74	-
5	Non-CRC	71	-
6	Non-CRC	81	-
7	Non-CRC	76	-
8	Non-CRC	62	-
9	Non-CRC	64	-
10	Non-CRC	67	

2.2 ISOLATION OF HUMAN NEUTROPHILS

To perform phenotypic and functional characterisation of circulating and tissue infiltrating human neutrophils, we set up two protocols of purification from peripheral blood and from intestinal specimens of IBD and CRC patients and of healthy individuals.

Peripheral blood samples and surgical specimens were received from Fondazione IRCCS Ca' Granda - Ospedale Maggiore Policlinico (Milan, Italy).

2.2.1 Isolation of human PMN from blood

10 ml of peripheral blood of CTR and IBD/CRC patients was mixed with an equal volume of dextran/saline solution (3% dextran from Leuconostoc spp (Sigma) in 0,9% NaCl and incubated in upright position for 20 minutes at room temperature.

Dextran promotes erythrocyte rouleaux formation, which results in differential sedimentation of RBC and the formation of a leukocyte-rich, RBC-poor plasma layer above the sedimented RBC. The upper layer (leukocyte-rich plasma), was aspirated and was centrifuged for 10 min at 1000 rpm, 5°C. Cell pellet was resuspended in a volume of 0,9% NaCl equal to the starting volume of blood and 15 ml Ficoll-Paque^{TM} PLUS (GE Healthcare) was statified beneath the cell suspension using a pipet centrifuged for 40 minutes at 1400 rpm, 20 °C, with no brake. After this stratification, the top (saline) layer was aspirated,as well as the Ficoll-Pacque layer, leaving the neutrophil/RBC layer.

To remove residual RBC, cells were subjected to hypotonic lysis by resuspending neutrophil/RBC pellet in 20 ml of cold 0,2% NaCl for exactly 30 seconds. At the end of this period, the isotonicity was restored by adding 20 ml of ice-cold 1,6% NaCl. This hypotonic lysis is based on the high sensitivity of RBC to hypotonicity, in comparison with neutrophils. However, the 30 seconds limit must be carefully observed since a more prolonged period of hypotonicity will result in neutrophil damage. After hypotonic lysis, cells were centrifuged for 6 minutes at 1000 rpm, 5°C, and the supernatant was discarded. Cells was resuspended RPMI (Fig. 10).

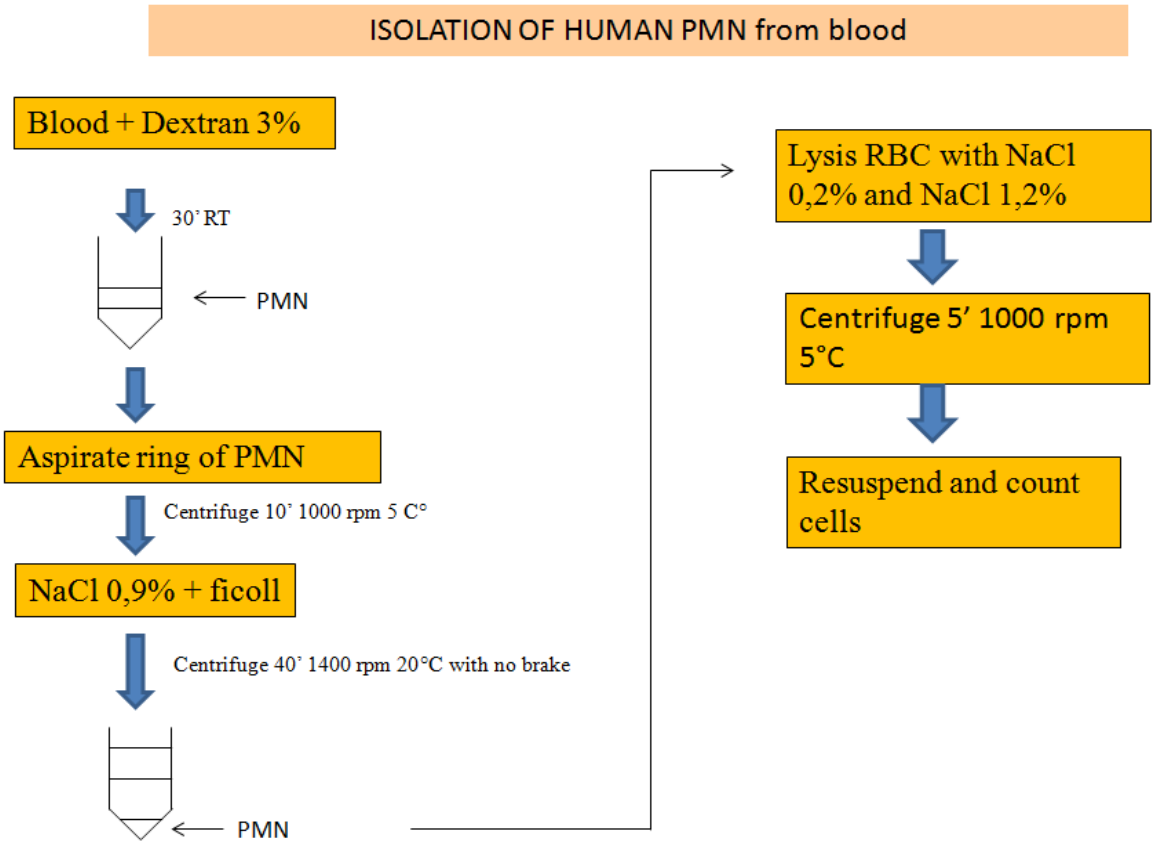

Figure 10. Schematic representation of isolation of human neutrophils from blood. Peripheral blood of CRC patients, IBD patients and healthy individuals, was diluted with 3% Dextran to remove RBC cells. Human neutrophils were separated from other blood cells by Ficoll.

2.2.2 Isolation of human PMN from Gut Tissue

To isolate neutrophils from intestinal surgical specimens, we firstly resected the tissue and separated the mucosa from the submucosa with forceps and surgical scissors. Gut mucosa was treated with DTT (Sigma Aldrich) solution for 15 min RT under slow rotation. Pretreatment with dithiothreitol (DTT) is necessary to dissolve intestinal mucus. After three washes with HBSS supplemented with antibiotics for 5 min, the mucosa was minced in small pieces with scalpel and knife and was incubated three times with EDTA solution for 50 min RT under slow rotation. EDTA was used to eliminate the epithelial cell layer. After three washes with HBSS supplemented with antibiotics for 5 min, the tissue was incubated in a digestion solution containing collagenase D (Roche) dissolved in RPMI 1640 medium sterile filtered (Gibco, Life Technologies) supplemented with 2% Penicillin/Streptomycin (Gibco, Life Technology) and Gentamycin (Roche, Ci= 40mg/ml) and 10% FBS (Gibco, life Technologies) at 37°C for 5 h in 5% CO_2 under slow rotation. Cells were filtered with a 100 µm cell strainer and then centrifuged at 1930 for 10 min at RT. The cells were purified using a Percoll gradient (100%, 60%, 40% and 30%) (Sigma) followed by centrifugation at 1930 RPM without accelerator nor brake for 30' RT to avoid cells mixed. Once collected, the cells were washed two times in PBS supplemented with 2% Penicillin/Streptomycin (Gibco, Life Technology) and Gentamycin (Roche, Ci= 40mg/ml), and the cells were counted using Burker counting chumber (Fig. 11).

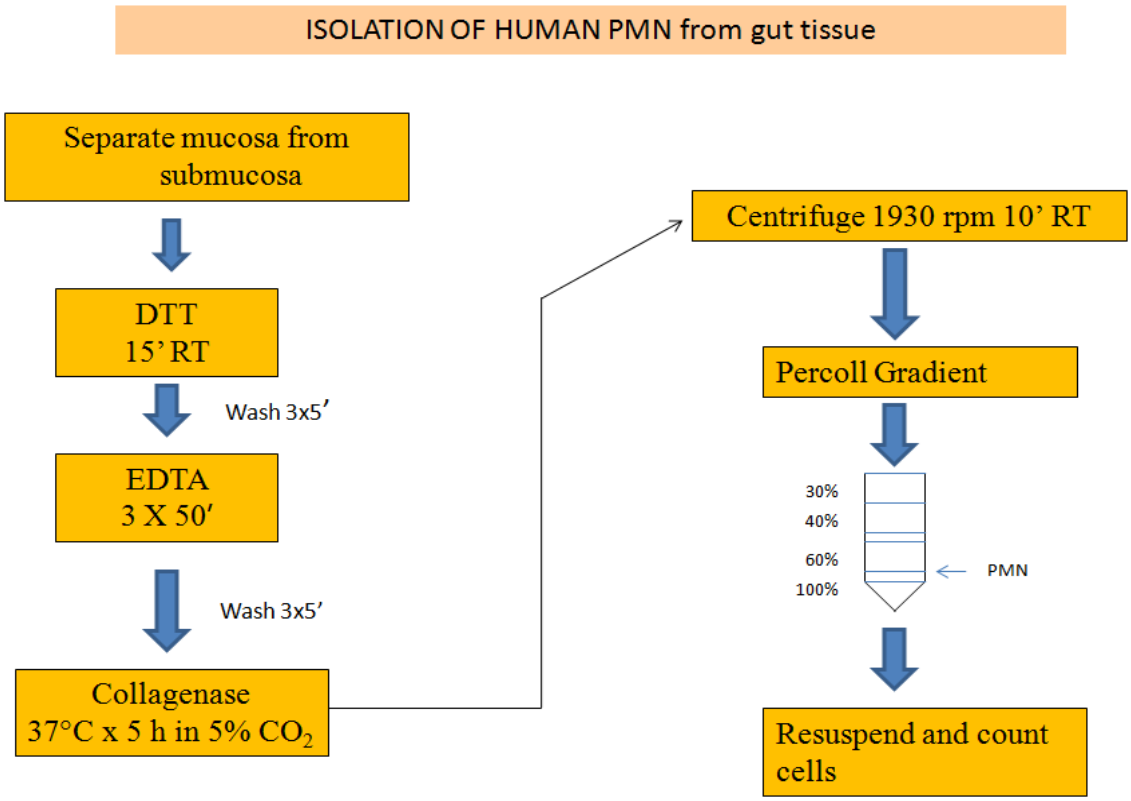

Figure 11. Schematic representation of isolation of human neutrophils gut tissue. Schematic representation of neutrophils isolation from human intestinal tissue.

2.2.3 Cell culture

Human neutrophils were cultured in RPMI 1640 medium sterile filtered (Gibco, Life Technologies) supplemented with 10% Fetal Bovine Serum (FBS) and 2% Penicillin/Streptomycin (Gibco, Life Technologies) and put in a humified incubator at 37°C with 5% CO_2

2.3 FLOW CYTOMETRY

2.3.1 Surface staining

After human neutrophils isolation, cells were collected in polypropylene tube and washed once with PBS (EuroClone). To phenotipically characterise the expression of surface markers on the different PMN population, cells were incubated with a cocktail of antibodies diluted in PBS for 20 minutes at 4°C and then centrifuged at 1500 RPM for 5 minutes. Pellet of human neutrophils were resuspended in PBS and flow cytometric analysis was performed. The antibodies described in table 5 were used:

Antibody	**Clone**	**Isotype**	**Vendor**
Anti-CD15	HI98	Mouse IgM, k	BD Bioscience
Anti-CD66b	G10F5	Mouse IgM, k	BioLegend
Anti-HLA-DR	AC122	Mouse,IgG2a	Miltenyi Biotec
Anti-CD80	2D10	Mouse, IgG1,k	Biolegend
Anti-CD86	IT2.2	Mouse IgG2b, k	BD Bioscience
Anti-PD-L1	MIH	Mouse, IgG1	BD Bioscience
Anti-CD1d	51.1	Mouse, IgG2b,k	BioLegend
Anti-CXCR4	12G5	Mouse,IgG2a	Miltenyi Biotec
Anti-CD62L	145/15	Mouse, IgG1,k	Miltenyi Biotec
Anti-CD163	GHI/61	Mouse, IgG1,k	BioLegend
Anti-CD200R	OX-108	Mouse, IgG1,k	BioLegend
Anti-CD54	HA58	Mouse, IgG1,k	BioLegend
Anti-CD206	DCN228	Mouse, IgG1	Miltenyi Biotec
Anti-CD301	REA586	Recombinant Human, IgG1	Miltenyi Biotec

Table 5. Anti-human antibodies used for surface stainings of human neutrophils cells.

To acquire and analyse cells, FACSCanto™ Cytometer (BD Bioscience) and BD FACSDiva™ software were used respectively.

2.3.2 Intracellular staining

To analyse cytokine profiles of human neutrophils, PMN cells were collected in 15 ml tube and washed once with PBS. After washing, cells were counted and fixed diluting 1 part of Fixation/ Permeabilization Concentrate with 3 parts of Fixation/Permeabilization Diluent (eBioscience, 100ul/ 10^6 cells) for 20 minutes at room temperature (RT). In order to permeabilize cell membranes, cells were resuspended with Permeabilization Buffer 10X containing 0,1% saponin (BD Bioscience) diluted 10-fold in distilled water and centrifuged at 1500 RPM for 5 minutes. Cells were stained with antibodies described in table 6 for 20 minutes at room temperature:

Antibody	**Clone**	**Isotype**	**Vendor**
Anti- IL-10	JES3-19F1	Rat IgG2a,k	BD Bioscience
Anti- IFN-y	48B3	Mouse IgG1,k	eBioscience

Table 6: Anti-human antibodies used for intracellular stainings of human neutrophils cells

After washing in permeabilization buffer, cells where resuspended in PBS and subsequently acquired on a BD FACSCanto™ (BD Bioscience) cytofluorimeter. Analysis was performed by using BD FACSDiva™ software.

2.4 HUMAN NEUTROPHILS STIMULATION

2.4.1 Luminal and mucusa-associated bacteria extraction

Human feces and mucus from gut of healthy controls and IBD and CRC patients were diluted with TES buffer containing 1M Tris-HCl, 5M NaCl and 0,5M EDTA and then centrifuged at 1200 RPM for 7 minutes. Samples were frozen at -80°C. In order to thaw cells, cryovials were incubated at 37 °C until they where thawed.

2.4.2 Bacterial infection and human PMN stimulation

Human PMN cells (300.000 cells/well) isolated from blood of CTR or IBD/CRC patients were incubated with luminal and mucus-associated-bacteria of gut into 96-well plates at 37 °C for 30 minutes. After this period, RPMI 1640 supplemented with 1% Penicillin/Streptomycin (Lonza) was added with final volume of 200 ul/well. In addition, the same number of cells were incubated with the respective TLR ligands in the same final volume. The final concentration of TLR ligands in the colture were: 0,2 ug/ml of LPS, 0,2 ug/ml Pam3 and 100 ug/ml of Poly(I:C). After incubation at 37°C, 5% CO_2, supernatants were collected for ELISA.

2.4.3 Cytokine determination by Enzyme Immunosorbent Assay (ELISA)

Released cytokines from human PMN assays, were measured by ELISA. ELISA plates (Maxisorp, Nunc) were coated with the capture antibodies over night (ON) at 4 °C, dissolved in PBS at a concentration from 1-10 ug/ml. The incubation of the coating antibody was done using 50 ul of antibody solution per well. After coating,

the ELISA plates were washed once with washing buffer containing PBS and 0,05% Tween 20 (Fluka) and wells were blocked with 100 ul of blocking buffer containing PBS, 0,05% Tween 20 and 1% bovine serum albumin (BSA, Sigma-Aldrich) for at least 1 hr with 50 ul of the sample or the standard recombinant cytokine. Then plates were whashed three times with washing buffer and incubated for at least 1hr with 50 ul of the detection antibody, diluted in blocking buffer at 1-5 ug/ml. After incubation plates were washed again three times and incubated with 60 ul of Streptavidin-HRP conjugate, Zymed) diluted in blocking buffer 1:4000. After 1 hr of incubation, plates were washed three times with washing buffer and incubated with 50 ul of the liquid substrate (3,3′,5,5′-Tetramethylbenzidine, TMB, ThermoFisher Scientific). The substrate incubation was done for 2 minutes and then stopped by addition of HCl . The adsorption was read at 450 nm, with ELISA reader (Plate Reader, Das). The samples were expressed as duplicates or triplicates, and the concentration of cytokines was calculated with a standard curve made by serial dilutions of the appropriate recombinant cytokine (Pharmigen).

The antibodies used for ELISA were described in table 7:

Antibody	**Clone**	**Isotype**	**Vendor**
Anti- IFN-Y	Purified,MD-1 Biotinylated 4S.B3	Mouse, IgG1,k Mouse IgG1,k	Biolegend
Anti-IL-10	Purified JES3-19F1 Biotinylated JES3-12G8	Rat IgG2a Rat IgG2a,k	Pharmigen Biolegend
Anti-IL-23	Purified MT86/221 Biotinylated MT155		MABTECH
Anti IL-12 (P70)	Purified 7B12 Biotinylated MT155	Rat IgG1	Biolegend

Table 7: Anti-human antibodies used for cytokines quantification

CHAPTER 3

AIMs OF THE PROJECT

As known, intestinal tissue of patients with active inflammatory bowel disease and colorectal cancer is heavily infiltrated by innate and adaptive immune cells, including granulocytes, macrophages and T and B lymphocytes. At present, the functional role of tissutal neutrophils in the modulation of mucosal immune responses in IBD and CRC is still largely unknown. Similarly, whether mucosal neutrophils play different roles in these two clinical conditions is still debated. In murine models of intestinal inflammation and cancer, a change in neutrophil phenotype and its possible functional consequence has already been described. However, there are limited data about the phenotype and function of neutrophil subsets in humans. In particular, the role of Tumor-associated neutrophils (TANs) in tumor development and promotion is beginning to be investigated in murine models, but it remains largely unexplored in humans.

On this basis, we focused our attention to circulating and tissutal human neutrophils isolated from patients with IBD, CRC patients and controls. Furthermore, we aimed to investigate the role of intestinal microbiota in the modulation of neutrophil functions.

The aims of this project are therefore to:

- Phenotipically characterize different human neutrophil subpopulations in IBD and CRC patients as compared with healthy individuals.

- Functional characterize PMN subpopulations in IBD and CRC, as evaluated by cytokine and ROS production

- To assess the response of IBD and CRC neutrophils to luminal and mucosa-associated bacterial stimulation.

- Determine whether a correlation exist between relative percentage of circulating and tissue-associated neutrophil subsets and therapy in IBD and CRC patients.

CHAPTER 4

RESULTS

Neutrophils play a critical role in tumor biology (Mantovani et al., 2011 and Piccard et al., 2012) and during intestinal chronic inflammation (Fournier et al., 2012), with different and often opposing functions. To explain these observations, it has been postulated the existence of distinct circulating and tissue-associated neutrophils subpopulations (Kolaczkowska E. et al., 2013).

4.1 Two subsets of neutrophils are identified in peripheral blood and intestinal tissue

Circulating human neutrophils are characterized in cancer patients by the expression of the molecules CD15 and by the upregulation of the molecule CD66b (Ilie M. et al., 2012). We used this marker combination to identify circulating neutrophils not only in intestinal cancer patients but also in IBD patients and in healthy individuals.
Moreover, we asked whether these markers might be useful to identify tissue-associated neutrophils in the lamina propria of IBD patients, of CRC patients and of healthy individuals (Fig.12).

By cytofluorimetric analysis we evaluated the frequency of $CD15^{+}CD66b^{+}$ neutrophils in blood and lamina propria (Fig.12).

We observed the existence of two distinct subsets of neutrophils on the basis of the expression levels of CD15 and CD66b both in blood (Fig. 12A) and in the intestinal tissue (Fig. 12C). The first subset was characterised by an increased expression of

CD66b. We will refer to this subset as $CD15^{+}CD66b^{high}$ throughout the thesis. The second subset was characterised by a lower expression of CD66b and we will refer to this subset as $CD15^{int}CD66b^{int}$ (Fig.12A,C).

The relative frequency of these two subpopulations in blood and in the lamina propria was clearly different (Fig.12B,D), with an enrichment of the $CD15^{+}CD66b^{high}$ in the gut tissue as compared to blood, where it was more abundant the $CD15^{int}CD66b^{int}$ population, especially in IBD and CRC patients (Fig. 12C).

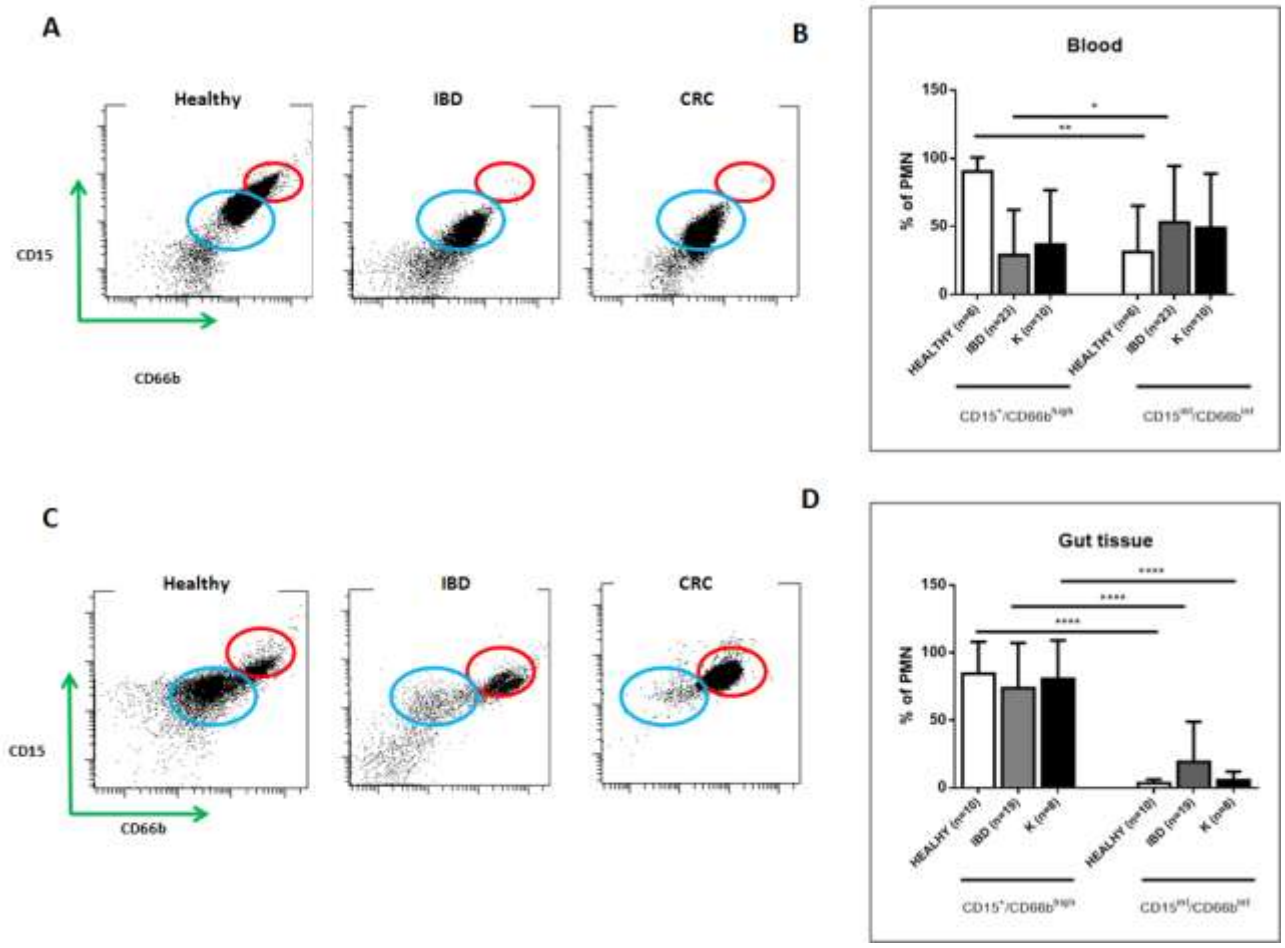

Fig 12. Human PMN subsets are identified on the basis of CD15 and CD66b expression. Representative dot plots of human PMN isolated from blood **(A)** and gut tissue **(C)**, and identified by the expression of CD15 and CD66b. **(B)** Data represent means ±SD of healthy (n=6), IBD (n=23) and CRC (n=10) patients in blood, and **(D)** healthy (n=10), IBD (n=19) and CRC (n=8) patients in gut tissue. ****p<0.0001, **p<0.01 and *p<0.05, Student's t-test.

4.2 Phenotypic characterization of $CD15^{+}/CD66b^{high}$ and $CD15^{int}/CD66b^{int}$ subpopulations by surface markers.

To further characterize the two neutrophil subsets identified in blood and intestinal tissue of patients and healthy individuals, we analysed a panel of surface molecules, known to be modulated according to the specific tissue distribution and effector functions (Z. G. Fridlender et al., 2009).

4.2.1 Expression of antigen presenting molecules: HLA-DR and CD1d

In addition to the phagocytic functions of neutrophils, PMN can also present antigens to $CD4^{+}$T cells (Radsak et al, 2000) and to non-conventional T cells (De Santo 2010). For these reasons, we evaluated the expression of surface molecules linked to T cell activation, such as HLA-DR (Fig. 13) and CD1d (Fig.14) that present antigens to $CD4^{+}$T cells and iNKT cells, respectively.

Interestingly, we observed an opposite trend between circulating and tissue-infiltrating neutrophil subsets expressing HLA-DR molecules. $HLA\text{-}DR^{+}$ neutrophils in blood were almost exclusively $CD15^{int}CD66b^{int}$, while in the lamina propria of healthy individuals and inside CRC tumours the vast majority of $HLA\text{-}DR^{+}$ neutrophils belonged to the $CD15^{+}CD66b^{high}$ subpopulation. A notable exception was observed in IBD patients, where HLA-DR+ cells neutrophils were, similarly to those circulating, mostly falling into the $CD15^{int}CD66b^{int}$ subset.

We observed that the overall HLA-DR expression is higher in the gut as compared to the blood (Fig. 13C,D). Moreover, the expression levels of HLA-DR did not differ between the two subsets. To note, in the mucosa of IBD patients, HLA-DR expression levels on the $CD15^{int}CD66b^{int}$ population was twice as high than that of $CD15^{+}CD66b^{high}$ neutrophil subset (Fig. 13C, D).

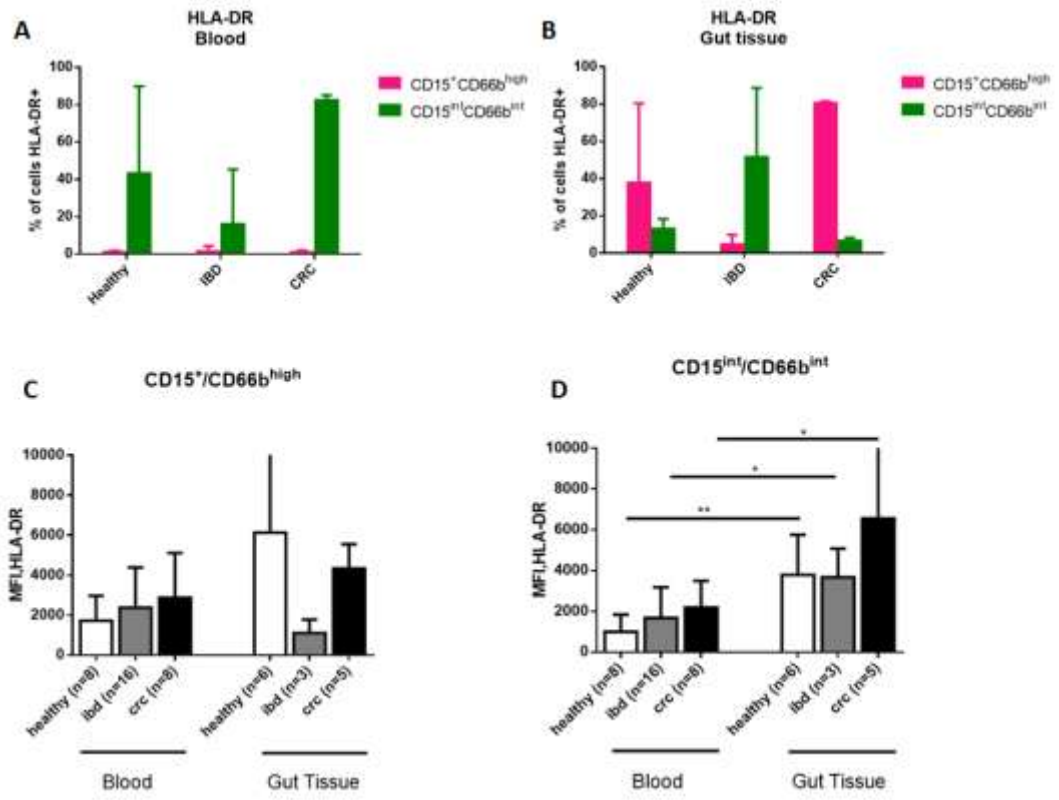

Fig. 13. HLA-DR is differentially expressed on circulating and tissue-resident neutrophil subsets. Frequency of HLA-DR^{+} neutrophil subsets in blood **(A)** and gut tissue **(B)** of IBD and CRC patients and healthy controls. Expression levels of HLA-DR (referred as Mean Fluorescence Intensity, MFI) on CD15^{+}CD66b^{high} (C) and CD15^{int}CD66b^{int} **(D)** neutrophils isolated from blood and gut tissue of IBD and CRC patients and healthy controls. Data represented as mean ±SD of healthy (n=8), IBD (n=16) and CRC (n=8) patients in blood, and healthy (n=6), IBD (n=3) and CRC (n=5) patients in gut tissue. **p<0.01 and *p<0.05, Student's t-test.

Similarly to what observed for HLA-DR, CD1d expression varied between circulating and tissue-associated neutrophils (Fig.14A, B). As for HLA-DR, CD1d^{+} neutrophils in blood were almost exclusively CD15^{int}CD66b^{int}, while in the lamina propria of IBD and CRC patients the majority of CD1d^{+} neutrophils were CD15^{+}CD66b^{high}; notably, in the lamina propria of healthy individuals no difference was observed between the two subsets of neutrophils concerning relative CD1d frequency (Fig. 14B).

As for HLA-DR, the two subsets did not show major differences in the expression levels of CD1d (Fig.14C,D). CD1d levels were generally higher in healthy individuals than in IBD-derived neutrophils. Conversely, inside the tumors both subsets expressed higher levels of CD1d, with the $CD15^{+}CD66b^{high}$ subset expressing the highest levels (Fig. 14C,D).

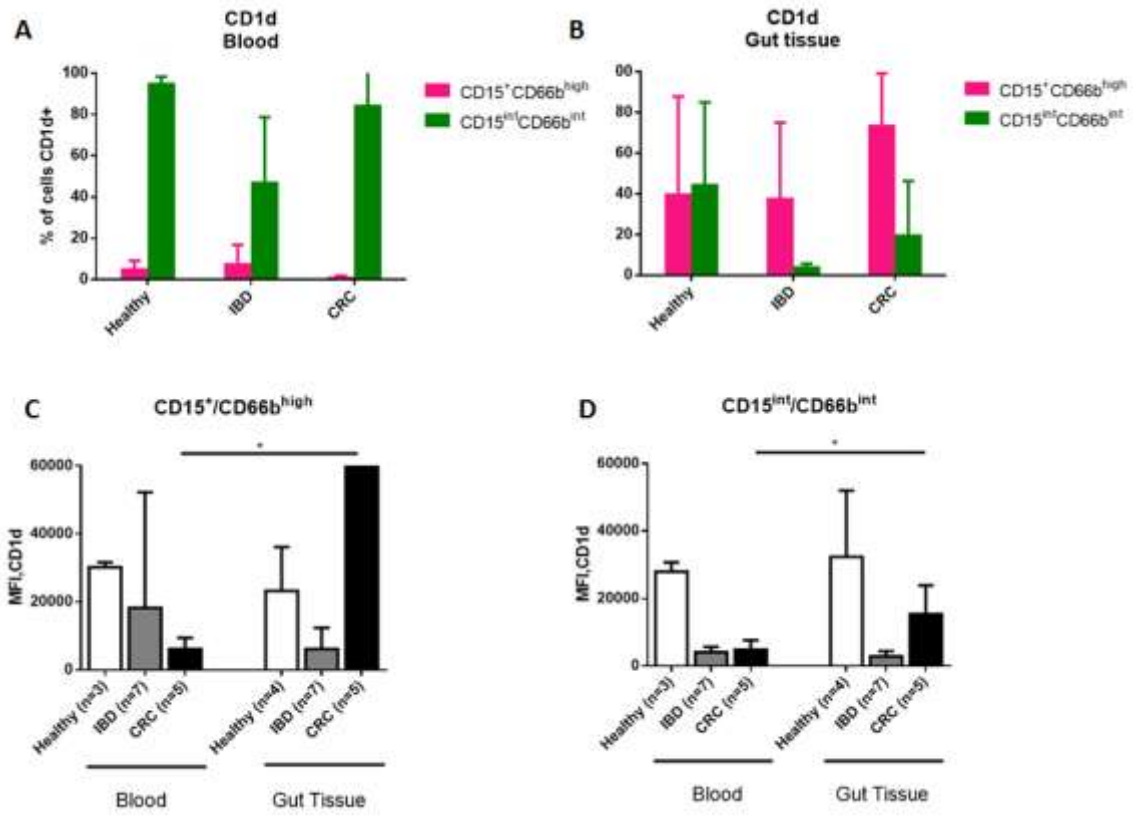

Fig.14. CD1d is differentially expressed on circulating and tissue-resident neutrophil subsets. Frequency of $CD1d^{+}$ neutrophil subsets in blood **(A)** and gut tissue **(B)** of IBD and CRC patients and healthy controls. Expression levels of CD1d (referred as Mean Fluorescence Intensity, MFI) on $CD15^{+}CD66b^{high}$ **(C)** and $CD15^{int}CD66b^{int}$ **(D)** neutrophils isolated from blood and gut tissue of IBD and CRC patients and healthy controls. CD1d. Data represented as mean ±SD of healthy (n=3), IBD (n=7) and CRC (n=5) patients in blood, and healthy (n=4), IBD (n=7) and CRC (n=5) patients in gut tissue. *$p<0.05$, Student's t-test.

4.2.2 Expression of co-stimulatory and inhibitory molecules: CD86 and PD-L1

Co-stimulatory and inhibitory molecules expression is associated with specific functional behaviours inside tissues (Gregory D. et al, 2013).

Thus, we investigated the expression patterns of the co-stimulatory molecule CD86 and the inhibitory molecule PD-L1 (also known as B7-H1) by the two subsets of human neutrophils, in blood and in intestinal tissue.

In line with the frequency of cells expressing antigen-presenting molecules in the different compartments, also CD86 was expressed by the vast majority of circulating $CD15^{int}CD66b^{int}$ neutrophils subpopulation (Fig.15A), while in the tissue it was mostly expressed by the $CD15^{int}CD66b^{int}$ one (Fig. 15B).

The expression levels of CD86 did not differ substantially between the two subsets (Fig.15C), although the circulating $CD15^{+}CD66b^{high}$ subpopulation expressed this co-stimulatory molecule at higher levels as compared to the gut (Fig.15D).

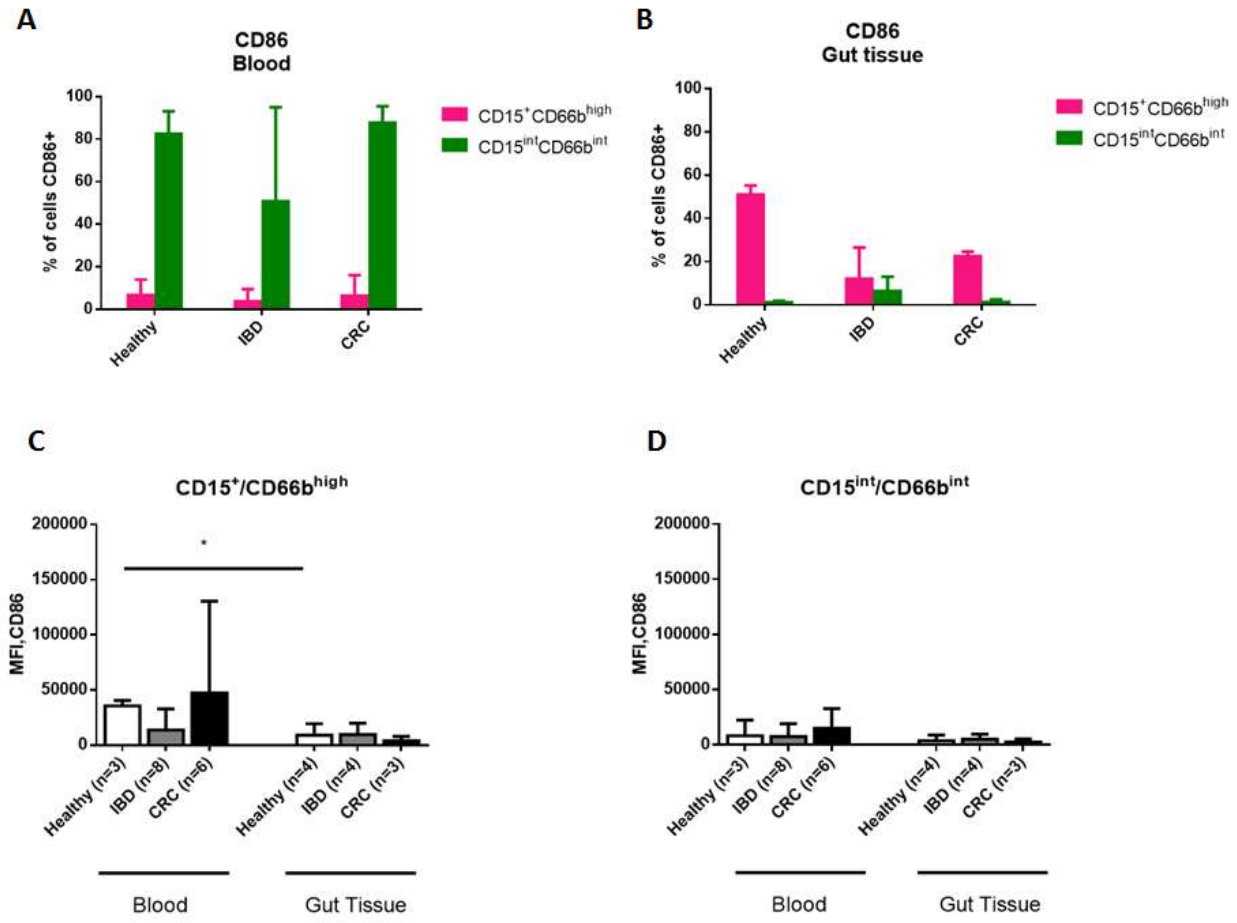

Fig. 15. CD86 is differentially expressed on circulating and tissue-resident neutrophil subsets . Frequency of $CD86^{+}$ neutrophil subsets in blood **(A)** and gut tissue **(B)** of IBD and CRC patients and healthy controls. Expression levels of CD86 (referred as Mean Fluorescence Intensity, MFI) on $CD15^{+}CD66b^{high}$ **(C)** and $CD15^{int}CD66b^{int}$ **(D)** neutrophils isolated from blood and gut tissue of IBD and CRC patients and healthy controls. Data represented as mean ± SD of healthy (n=3), IBD (n=8) and CRC (n=6) patients in blood, and healthy (n=4), IBD (n=4) and CRC (n=3) patients in gut tissue. *$p<0.05$, Student's t-test.

PD-L1 was expressed almost exclusively by $CD15^{int}CD66b^{int}$ circulating neutrophils isolated from healthy individuals and from IBD patients, while surprisingly by very few of those isolated from CRC patients (Fig. 16A).

In the gut, the differences between the two subpopulations were almost absent in terms of frequency. Interestingly, PD-L1 was expressed by a higher fraction of neutrophils isolated from healthy individuals as compared to IBD or to CRC patients (Fig.16B).

The general expression levels of PD-L1 were higher in the $CD15^{+}CD66b^{high}$ subset as compared to the intermediate, but we did not observe striking differences between groups of patients and healthy individuals (Fig.16C,D).

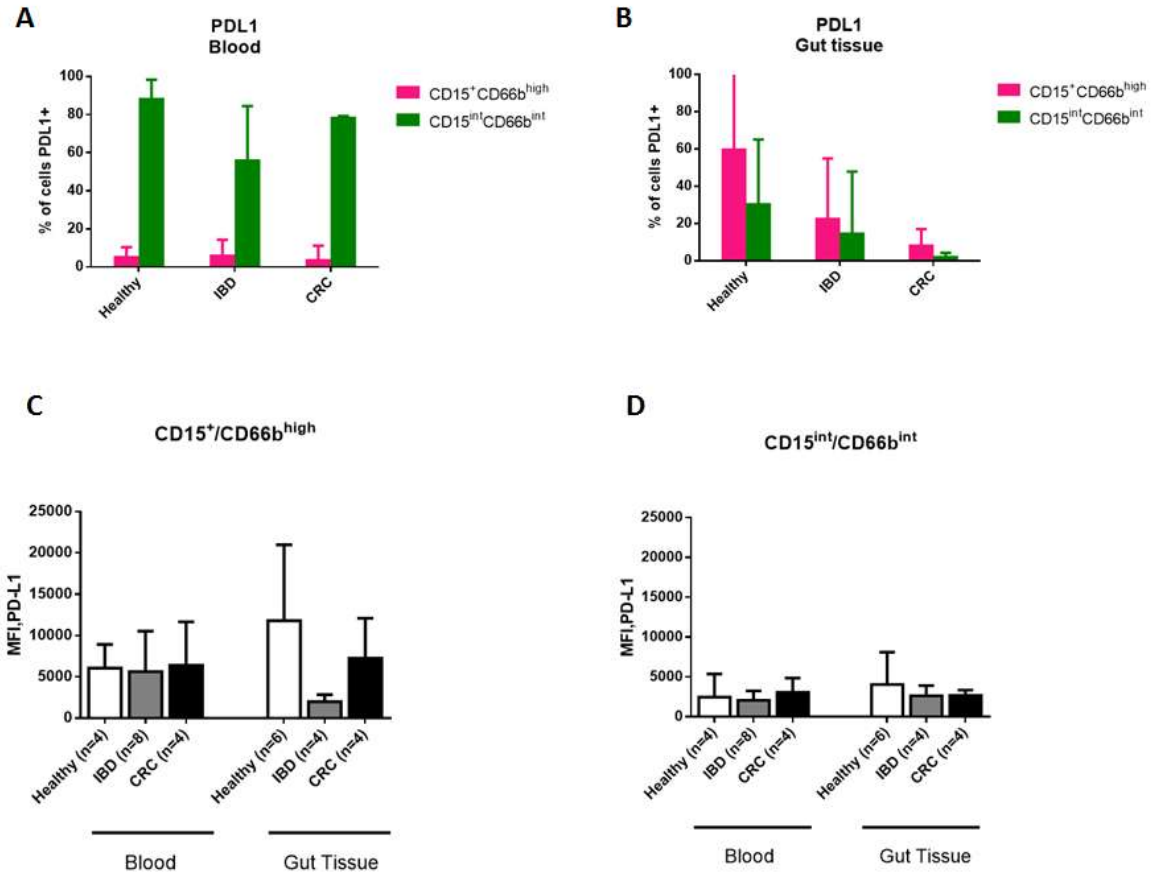

Fig. 16. PD-L1 is differentially expressed on circulating and tissue-resident neutrophil subsets. Frequency of PD-L1^{+} neutrophil subsets in blood **(A)** and gut tissue **(B)** of IBD and CRC patients and healthy controls. Expression levels of PD-L1 on CD15^{+}CD66b^{high} **(C)** and CD15^{int}CD66b^{int} **(D)** neutrophils isolated from blood and gut tissue of IBD and CRC patients and healthy controls. Data represented as mean± SD of healthy (n=4), IBD (n=8) and CRC (n=4) patients in blood, and healthy (n=6), IBD (n=4) and CRC (n=4) patients in gut tissue.

4.2.3 Expression of molecules involved in neutrophil trafficking: CXCR4, CD54 and CD62L

Neutrophil recruitment to the site of injury is an essential first step of an anti-bacterial response, but it is also essential for tumor infiltration (Kolaczkowska E. at al, 2013). Thus, we evaluated the possibility that the two neutrophils subsets isolated from IBD and/or CRC patients might manifest modifications in the expression pattern of molecules involved in trafficking.

We selected CXCR4, which is expressed upon inflammatory stimuli (Christoffersson et al., 2012) and is overexpressed in human cancers (Uhlen,& Rhim, 2010), CD54 (also known as ICAM-1) which is important for neutrophil transmigration through the endothelial monolayer, and CD62L also known as L-selectin, that facilitates infiltration of neutrophils into the site of inflammation.

As expected for a homing molecule, CXCR4 was expressed at higher levels in the tissues as compared to the blood (Fig.17A,B) but we did not observed a different expression between the two subpopulations.

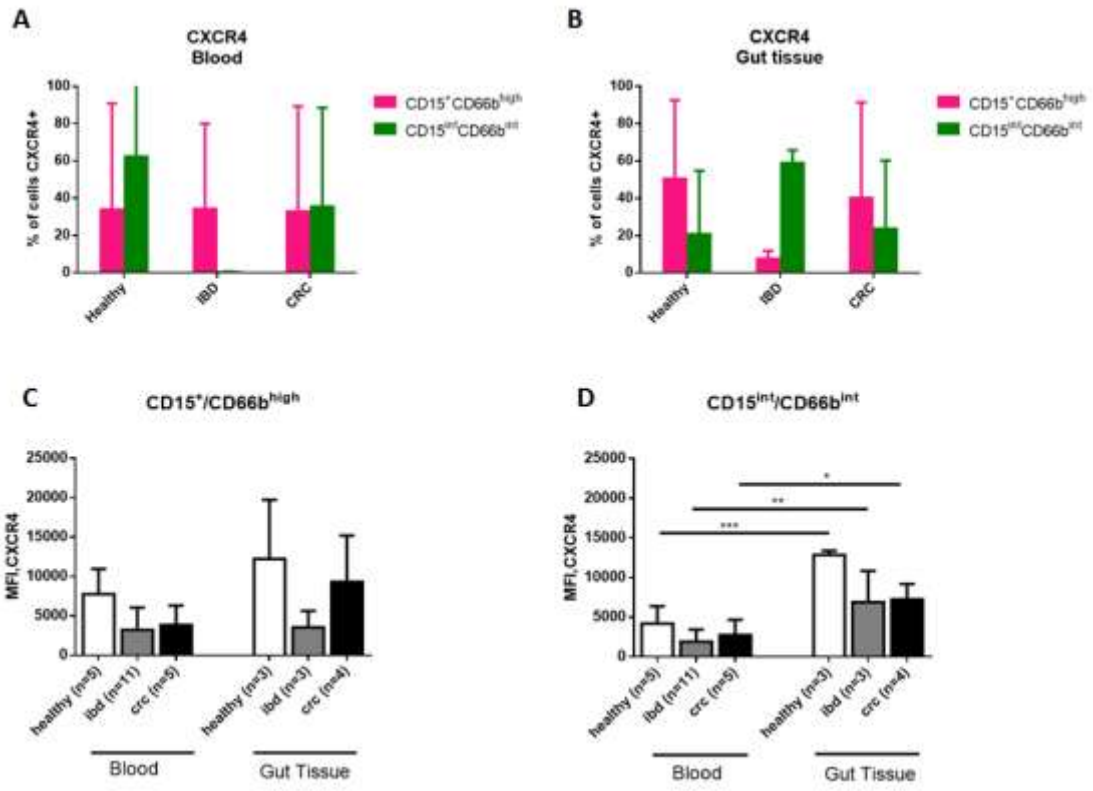

Fig. 17. CXCR4 is differentially expressed on circulating and tissue-resident neutrophil subsets. Frequency of $CXCR4^{+}$ neutrophil subsets in blood **(A)** and gut tissue **(B)** of IBD and CRC patients and healthy controls. Expression levels of CXCR4 on $CD15^{+}CD66b^{high}$ **(C)** and $CD15^{int}CD66b^{int}$ **(D)** neutrophils isolated from blood and gut tissue of IBD and CRC patients and healthy controls. Data represented as mean ± SD of healthy (n=3), IBD (n=11) and CRC (n=5) patients in blood, and healthy (n=3), IBD (n=3) and CRC (n=4) patients in gut tissue. ***p<0.001, **p<0.01 and *p<0.05, Student's t-test.

CD54 was expressed by circulating neutrophils isolated from IBD and CRC patients, but it was mostly absent on the surface of neutrophils isolated from healthy individuals (both circulating and in the gut) and no differences were observed among the two sub-populations. Conversely, CD54 was expressed in the gut almost exclusively by $CD15^{+}CD66b^{high}$ cells (Fig. 18B). The expression levels of CD54 were

higher in IBD and CRC patients as compared to healthy individuals, with no significant differences between the two subpopulations (Fig. 18C,D).

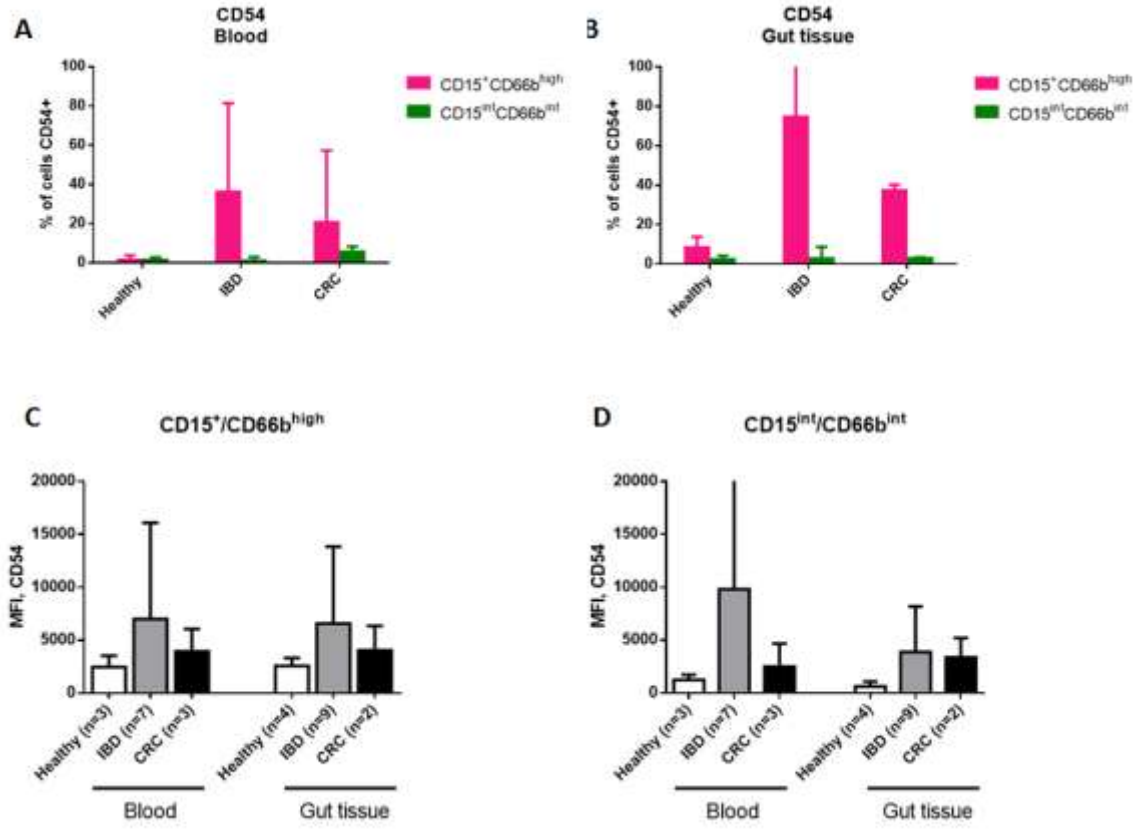

Fig. 18. CD54 is differentially expressed on circulating and tissue-resident neutrophil subsets. Frequency of $CD54^{+}$ neutrophil subsets in blood **(A)** and gut tissue **(B)** of IBD and CRC patients and healthy controls. Expression levels of CD54 on $CD15^{+}CD66b^{high}$ **(C)** and $CD15^{int}CD66b^{int}$ **(D)** neutrophils isolated from blood and gut tissue of IBD and CRC patients and healthy controls. Data represent means ± SD of healthy (n=3), IBD (n=7) and CRC (n=3) patients in blood, and healthy (n=4), IBD (n=9) and CRC (n=2) patients in gut tissue.

Finally, CD62L was expressed exclusively by circulating $CD15^{int}CD66b^{int}$ neutrophils of IBD and CRC patients, but it was expressed only on a small fraction of neutrophils of healthy individuals. In the gut, as it was observed for other surface molecules, $CD15^{+}CD66b^{high}$ cells from IBD and CRC patients were the cells mostly expressing CD62L (Fig. 19A,B).

Interestingly, expression levels of CD62L were variable on the surface of neutrophils, although $CD15^{int}CD66b^{int}$ cells isolated from the lamina propria expressed higher levels of CD62L than their circulating counterpart.

Moreover, $CD15^{+}CD66b^{high}$ cells isolated from healthy individuals and CRC patients seemed to express higher levels of CD62L as compared to those isolated IBD patients.

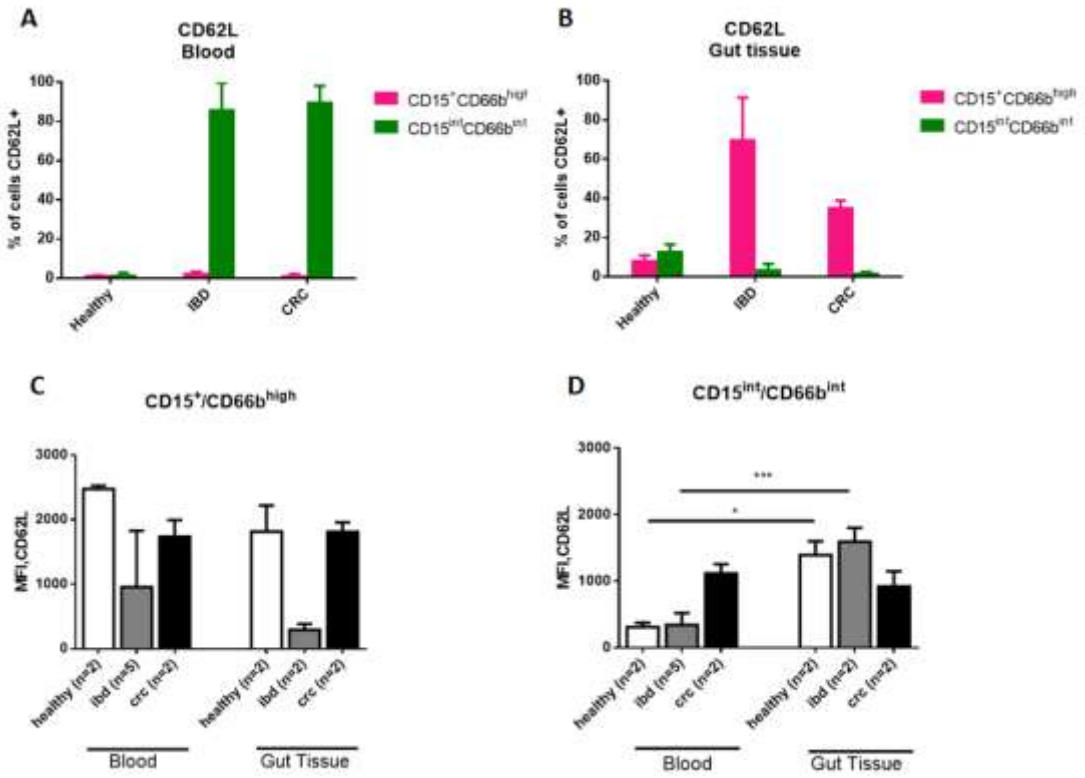

Fig.19. CD62L is differentially expressed on circulating and tissue-resident neutrophil subsets. Frequency of $CD62L^{+}$ neutrophil subsets in blood **(A)** and gut tissue **(B)** of IBD and CRC patients and healthy controls. Expression levels of CD62L on $CD15^{+}CD66b^{high}$ **(C)** and $CD15^{int}CD66b^{int}$ **(D)** neutrophils isolated from blood and gut tissue of IBD and CRC patients and healthy controls. Data represented as mean± SD of healthy (n=2), IBD (n=5) and CRC (n=2) patients in blood, and healthy. (n=2), IBD (n=2) and CRC (n=2) patients in gut tissue. ***p<0.001 and *p<0.05, Student's t-test.

4.2.4 Clinical correlation

Since we observed different frequencies of $CD15^{+}CD66b^{high}$ and $CD15^{int}CD66b^{int}$ subsets in blood and intestinal tissue of IBD and CRC patients, we asked whether immunomodulators could play a role in producing these alterations. Indeed, therapeutic regimens were highly different among patients, as shown in Tables 1,2,3 and 4 of the material and methods section.

We decided therefore to perform separate analyses on the patients based upon whether they were receiving or not at the time of surgical intervention. Immunosuppressants include thiopurines such as 6-mercaptopurine (6-MP), azathioprine (AZA), steroids and anti-TNF (infliximab or adalimumab). As depicted in Fig. 20, We observed that both circulating PMN subsets decreased in IBD and CRC patients under immunosuppressive treatment.

On the contrary, in the intestinal mucosa, the frequencies of the $CD15^{+}CD66b^{high}$ population slightly increased in IBD and CRC patients under immunosuppressive therapy while the percentage of the $CD15^{int}CD66b^{int}$ subset strongly decreased in IBD and CRC patients under immunosuppressive treatment.

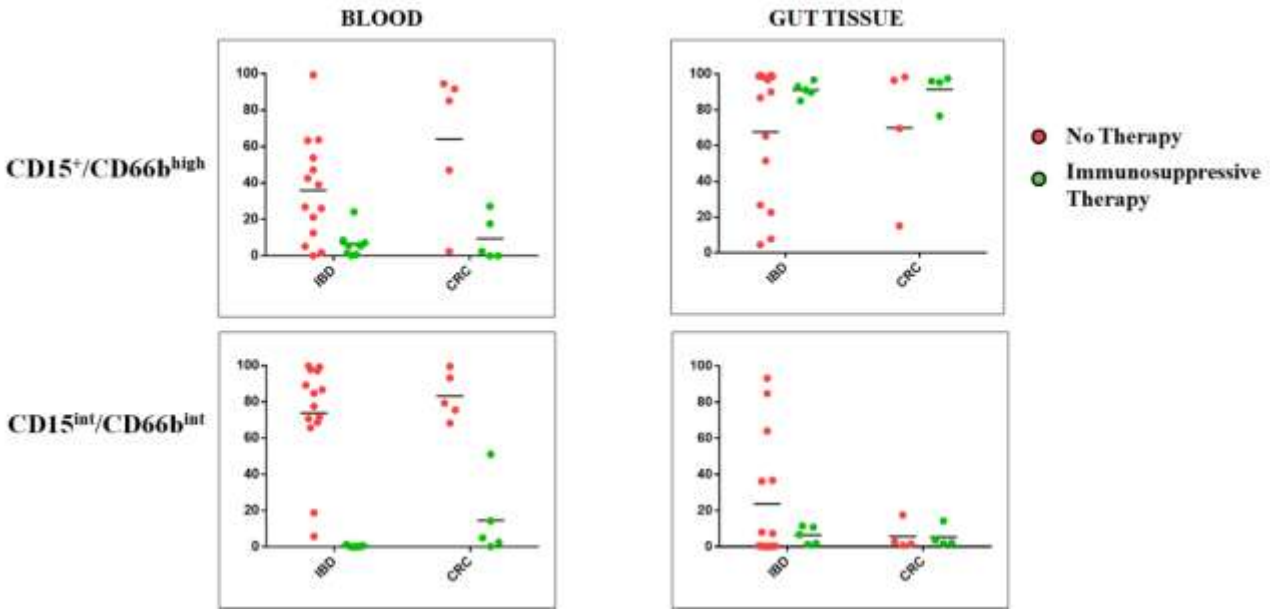

Fig.20. Immunosuppressive therapy impacts on PMN frequency in blood and gut tissue. Scatter dot plot represent the percentage of PMN subsets in blood and gut tissue of IBD and CRC patients with (in red) or without (in green) immunosuppressive therapy ongoing.

4.2.4.1 Antigen presenting molecules expression and clinical correlation

Next, we correlated the expression of antigen presenting molecules on the surface of the two PMN subsets in blood and intestinal tissue of IBD and CRC patients with or without immunosuppressive treatment (Fig.21).

HLA-DR expression on the $CD15^{+}CD66b^{high}$ subset did not correlate with ongoing therapy. On the contrary, it did correlate in IBD patients, demonstrating a strong increase of $HLA\text{-}DR^{+}$ cells among $CD15^{int}CD66b^{int}$ subsets in patients under immunosuppressive therapy (Fig 21A).

CD1d expression on both subsets and similarly in IBD and CRC patients was decreased in the intestinal tissue of patients undergoing immunosuppressive therapy (Fig 21B).

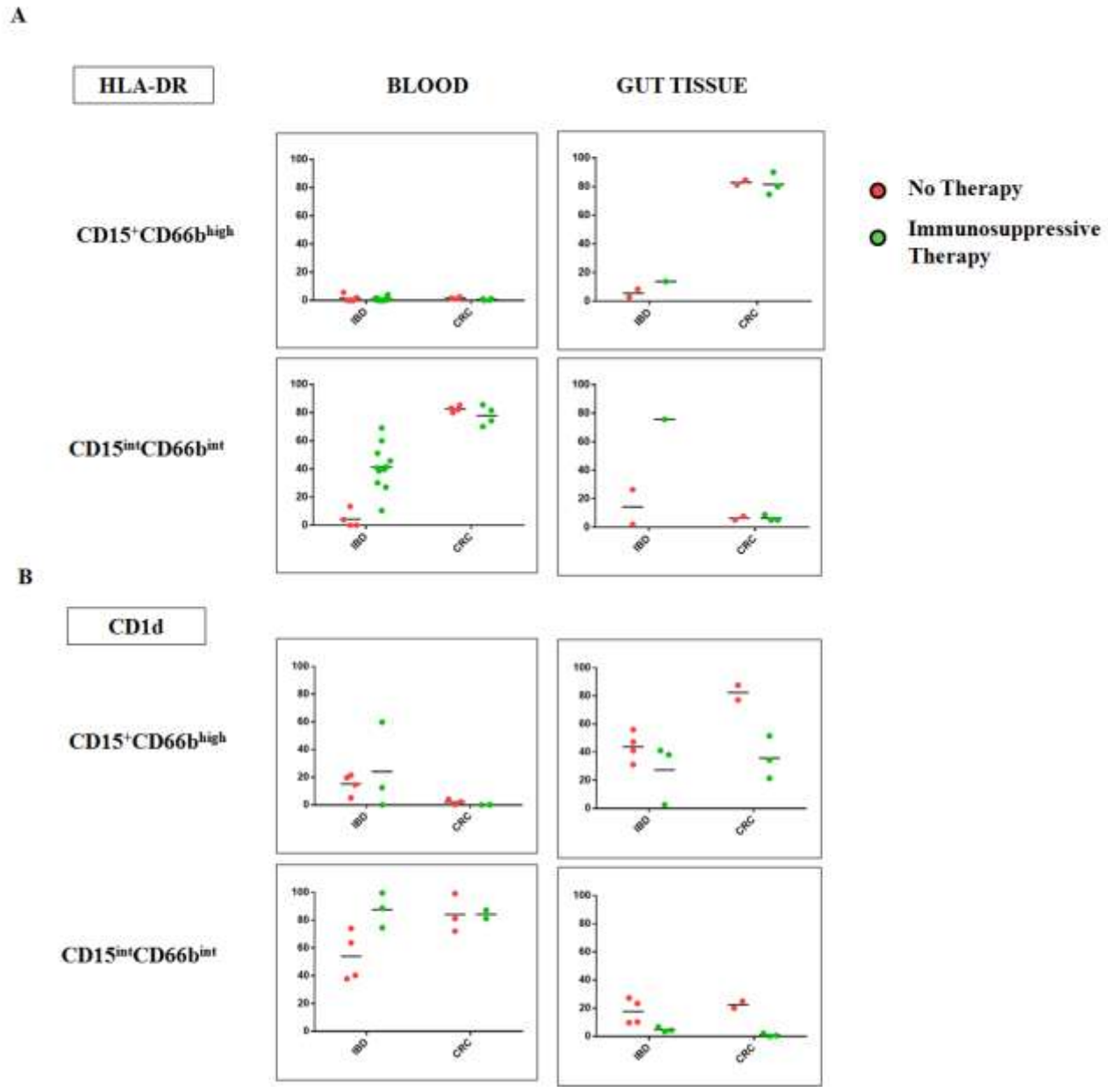

Fig.21. Immunosuppressive therapy increases HLA-DR expression on CD15^{int}CD66b^{int} neutrophils, of IBD patients. Scatter dot plot represent the percentage of PMN subsets expressing HLA-DR **(A)** and CD1d **(B)** in blood and gut tissue of IBD and CRC patients with (in red) or without (in green) immunosuppressive therapy.

4.2.4.2 Activator and inhibitory molecules and clinical correlation

Next, we correlated the expression of CD86 and PD-L1 on the surface of the two PMN subsets in blood and intestinal tissue of IBD and CRC patients with or without immunosuppressive treatment ongoing (Fig 22).

CD86 expression on the $CD15^{+}CD66b^{high}$ subset increased in blood and in tissue of IBD and CRC patients undergoing immunosuppressive therapy (not in CRC patients gut, though) (Fig 22A). Similarly, CD86 expression on the $CD15^{int}CD66b^{int}$ subset strongly increased in the blood of IBD patients under therapy, but not in the gut and no differences were observed in CRC patients.

PD-L1 expression, instead, was strongly reduced on the surface of both circulating and infiltrating $CD15^{+}CD66b^{high}$ neutrophils (Fig 22B) in both IBD and CRC patients under immunosuppressive therapy. PD-L1 expression was lower on circulating $CD15^{int}CD66b^{int}$ from IBD patients undergoing immunosuppressive therapy, but it was unchanged in the gut of both IBD and CRC patients.

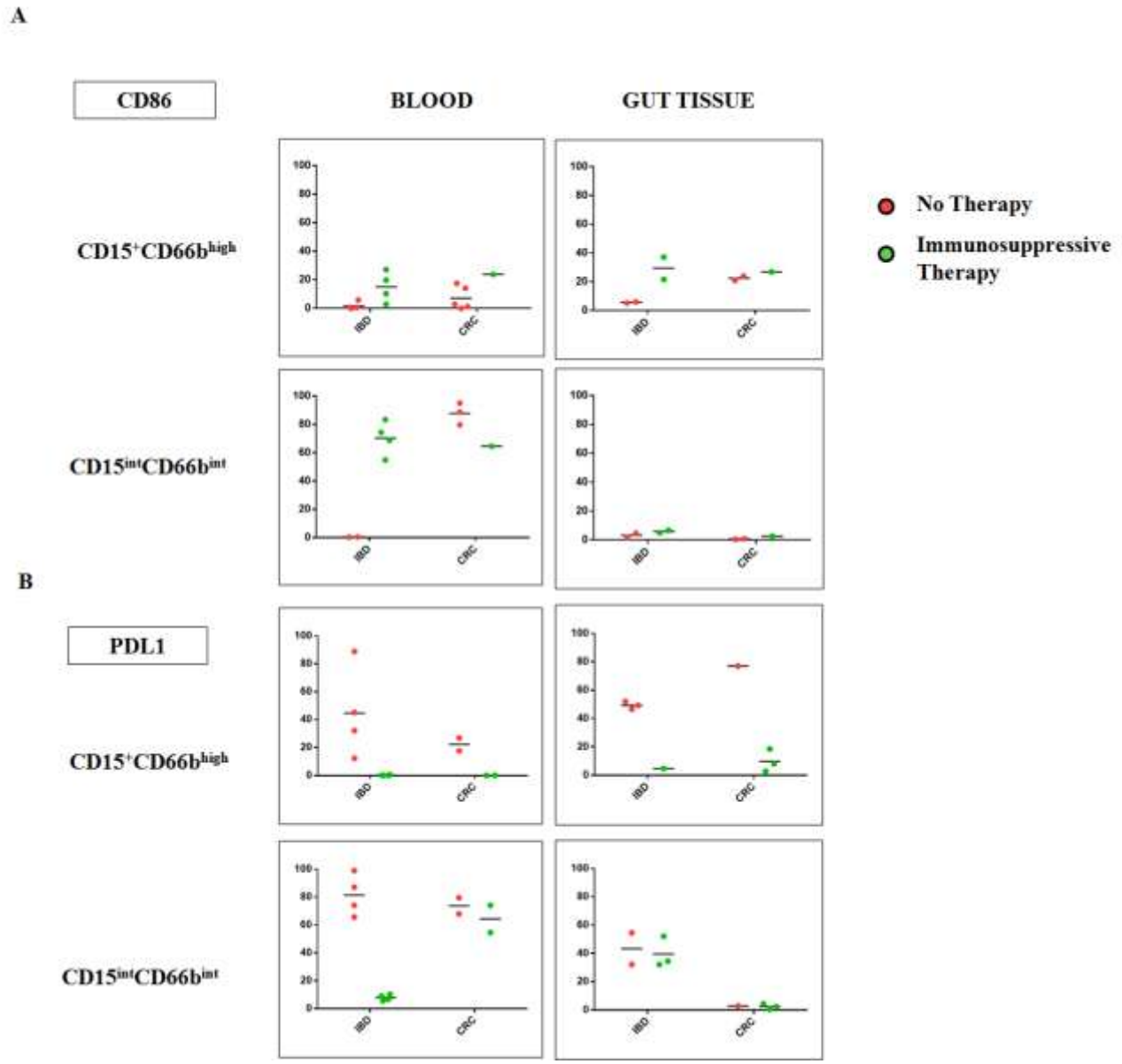

Fig.22. Immunosuppressive therapy differently impacts on CD86 and PD-L1 expression of IBD and CRC PMN. Scatter dot plot represent the percentage of PMN subsets expressing CD86 **(A)** and PD-L1 **(B)** in blood and gut tissue of IBD and CRC patients with (in red) or without (in green) immunosuppressive therapy ongoing.

4.2.4.3 Expression of molecules involved in trafficking and clinical correlation

Next, we correlated the expression of molecules involved in the migration of neutrophils on the surface of the two PMN subsets in blood and intestinal tissue of IBD and CRC patients with or without immunosuppressive treatment ongoing (Fig. 23).

CXCR4 expression on the $CD15^{+}CD66b^{high}$ subset decreased in blood and increased in tissue of IBD and CRC patients undergoing immunosuppressive therapy (Fig. 23A). On the contrary, CXCR4 expression on the $CD15^{int}CD66b^{int}$ subset was increased in the blood of IBD patients under therapy, but not in the gut and no differences were observed in CRC patients.

CD54 expression, instead, was strongly reduced on the surface of both circulating subsets in both IBD and CRC patients under immunosuppressive therapy, and it was slightly increased on the surface of the $CD15^{int}CD66b^{int}$ neutrophils in the gut of IBD patients (Fig.23B).

CD62L expression on circulating neutrophils was unaffected by immunosuppressive therapy, but it was decreased on the surface of gut $CD15^{int}CD66b^{high}$ neutrophils in both IBD and CRC patients under immunosuppressive therapy and it was slightly increased on the surface of the $CD15^{int}CD66b^{int}$ neutrophils in the gut of IBD patients (Fig.23C).

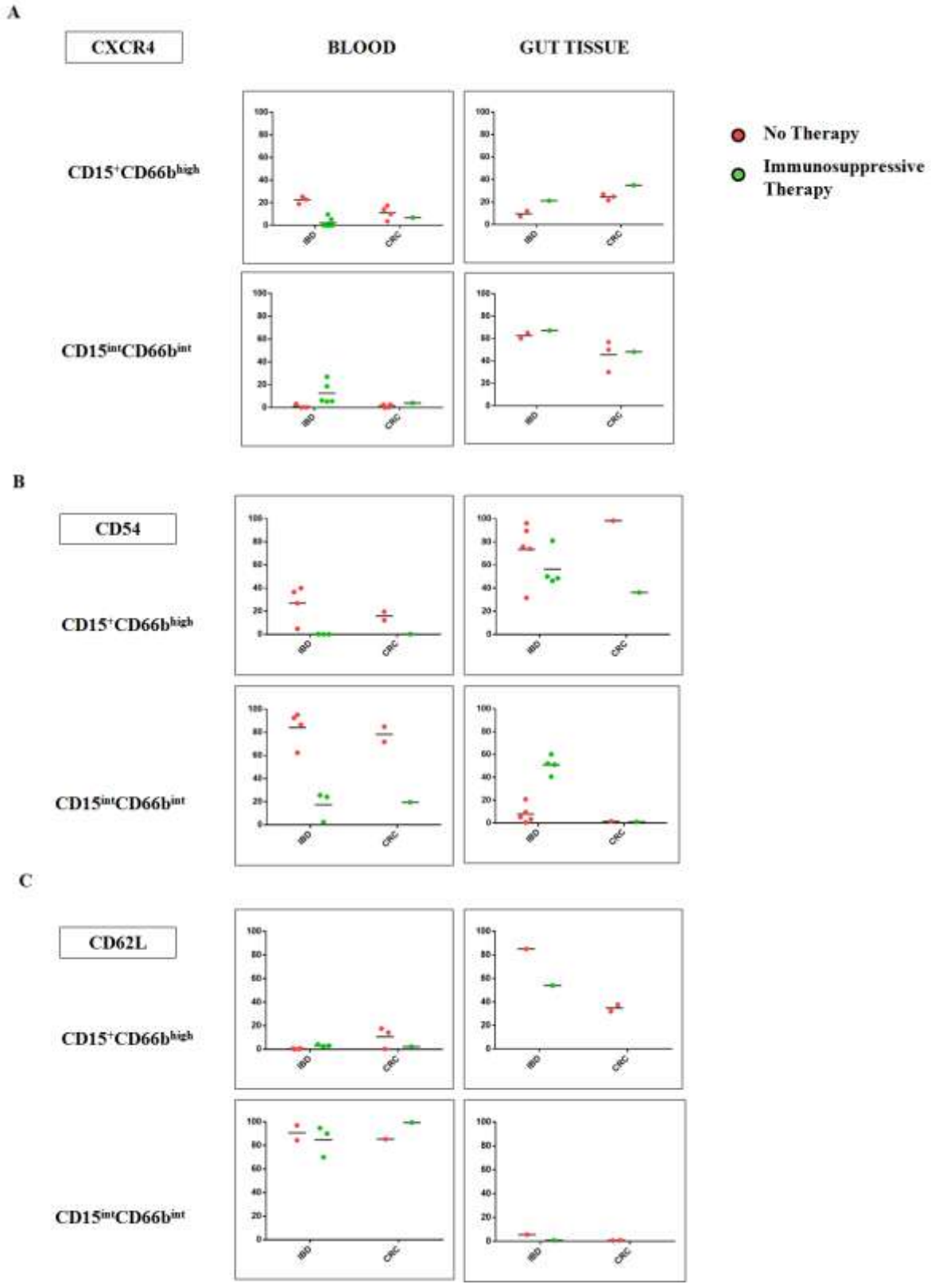

Fig.23. Immunosuppressive therapy modulates CXCR4, CD54 and CD62L expression on PNM subsets of IBD and CRC patients. Scatter dot plot represent the percentage of PMN subsets expressing CXCR4 **(A)**, CD54 **(B)** and CD62L **(C)** in blood and gut tissue of IBD and CRC patients without treatment (in red) and during immunosuppressive therapy (in green).

4.2.5 Expression of molecules involved in neutrophil N1/N2 polarization

Recent reports (Virna Cortez-Retamozo et al., 2012) demonstrated that neutrophils, like macrophages, may have the ability to differentiate into two distinct functional subsets: one more inflammatory/anti-tumorigenic (N1) and a second more anti-inflammatory/protumorigenic (N2).

To evaluate if $CD15^{+}CD66b^{high}$ and $CD15^{int}CD66b^{int}$ subsets in blood and gut tissue of IBD and CRC patients showed a skewed N1/N2, we phenotipically characterized neutrophil subsets through the expression of known N1 and N2 markers, similarly to those identified for M1 and M2 macrophages (Subhra KB at al., 2010).

Among others, classical N1 markers are HLA-DR and CD86, that we evaluated separately in sections 4.2.1 (HLA-DR) and 4.2.2 (CD86). In this section we will evaluate the co-expression of these N1 markers on the surface of the identified neutrophils subpopulations.
Typical N2 markers instead are CD301, CD206, CD163 and CD200R (Koning N. at al., 2010). In this section we will evaluate these markers separately and then, as for N1 markers, we will evaluate their co-expression on the two subsets of neutrophils.

4.2.5.1 Co-Expression of molecules involved in N1 differentiation (HLA-DR and CD86)

As it was observed in Figures 13 (HLA-DR) and 15 (CD86), the expression of these two molecules linked to an inflammatory phenotype are associated to the $CD15^{int}CD66b^{int}$ population in blood and to the $CD15^{+}CD66b^{high}$ in the gut.

Similarly, these N1 molecules were co-expressed by $CD15^{+}CD66b^{high}$ neutrophils in the gut (Fig 24). Unexpectedly, IBD patients $CD15^{+}CD66b^{high}$ neutrophils were "less" polarised towards an N1 phenotype as compared to CRC patients or healthy controls.

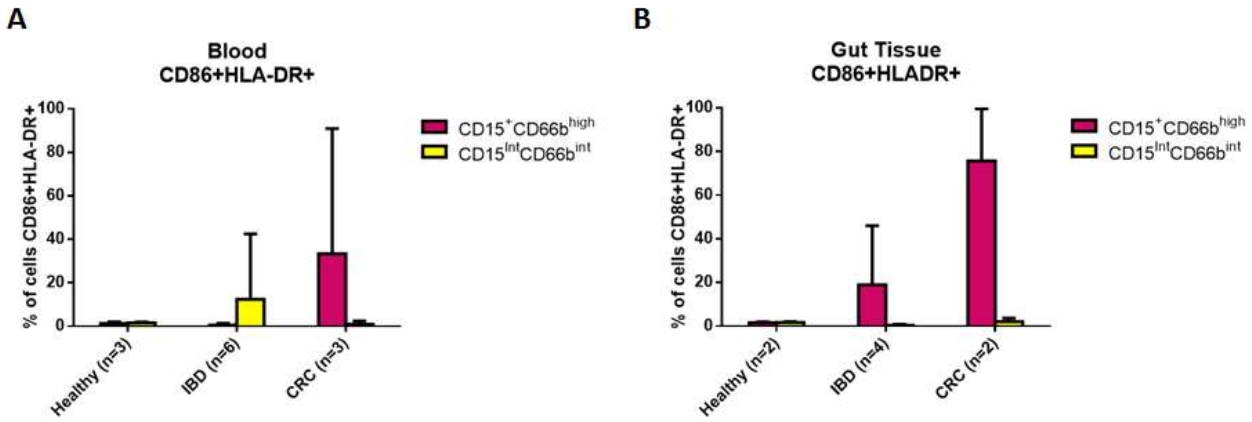

Fig.24. Human PMN subsets expressing a N1 phenotype. Histograms showing the percentage of $CD15^{+}CD66b^{high}$ and $CD15^{int}CD66b^{int}$ with N1 phenotype in blood and gut tissue of IBD and CRC patients and controls.

4.2.5.2 Expression of molecules involved in N2 polarization: CD163, CD301, CD206 and CD200R

CD163 is a member of the scavenger receptor (Onofre G., 2009). This scavenger receptor is selectively expressed on cells of monocytes and macrophages lineage. This molecule participates in adhesion to endothelial cells, in tolerance induction and tissues regeneration exhibiting strong anti-inflammatory properties (van Gorp H. et al., 2010).

CD301, also known as CLEC10A, is a transmembrane glycoprotein that is expressed on immature myleloid dendritic cells and alternatively activated (tolerogenic) macrophages (Raes G. et al., 2005). Human CD301 has an exclusive specificity for rare terminal GalNAc structures, which are revealed on the tumor-associated mucin MUC1 and CD45. This interaction inhibits effector T cell activation and induces their apoptosis.

CD206, also known as mannose receptor (MR), is a type I membrane protein that is expressed on different types of cells such as macrophages and dendritic cells (Verreck FA. et al., 2006). This molecule recognizes a range of microbial carbohydrates bearing mannose, fucose, or N-acetyl glucosamine, sulfated carbohydrate antigens and collagens. CD206 mediates endocytosis and phagocytosis as well as activation of macrophages and antigen presentation. Recently, CD206 on lymphatic endothelial cells was found to be involved in leukocyte trafficking and a contributor to the metastatic behavior of cancer cells. It suggests that this molecule may be a potential target in controlling inflammation and cancer metastasis by targeting the lymphatic vasculature.

CD200R, also known as OX2R, is a membrane glycoprotein expressed primarily by monocytes and neutrophils but also by other leukocytes including T lymphocytes and

mast cells (Wright GJ et al., 2003). The interaction between CD200 and CD200R may contribute to pathways that suppress and limit macrophage induced inflammatory damage in tissue.

Analysis of CD163 revealed a more prominent expression by tissue-derived as compared to circulating neutrophils (Fig 25A,B), and by the $CD15^{+}/CD66b^{high}$ subpopulation as compared to $CD15^{int}CD66b^{int}$ population, especially by neutrophils isolated from IBD and CRC patients (Fig.25A,B).

To note, expression levels are higher in IBD-derived neutrophils, regardless the subpopulation (Fig 25C,D)

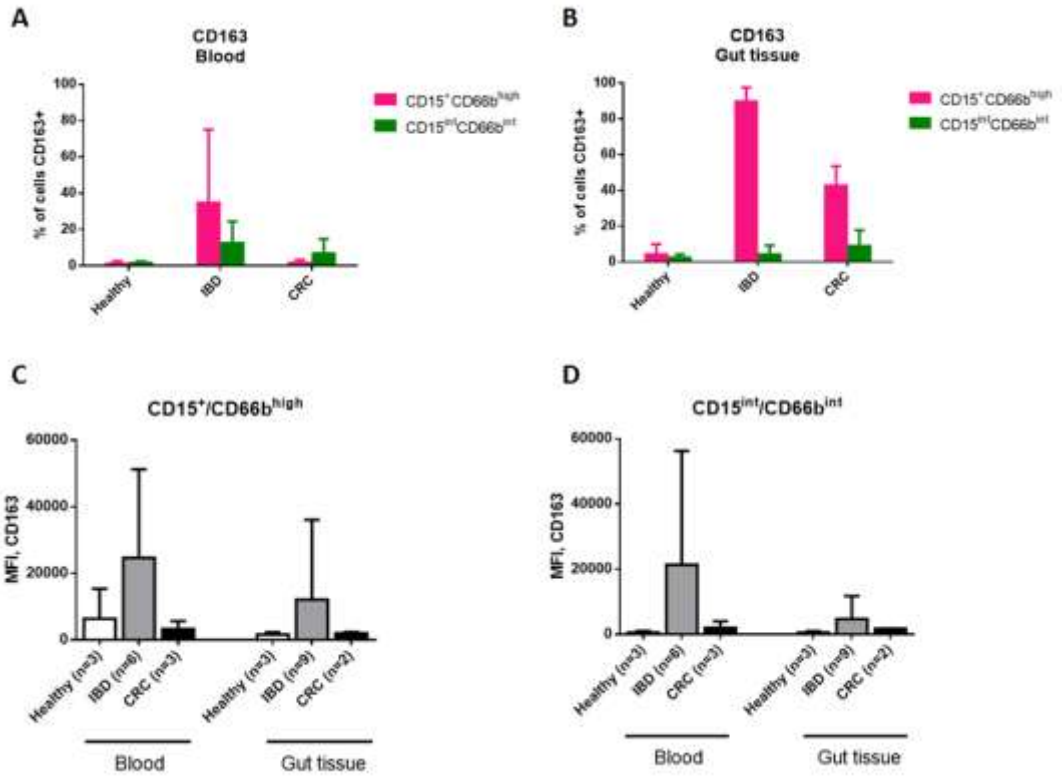

Fig.25. CD163 is differentially expressed on circulating and tissue-resident neutrophil subsets. Frequency of $CD163^{+}$ neutrophil subsets in blood **(A)** and gut tissue **(B)** of IBD and CRC patients and healthy controls. Expression levels of CD163 (referred as Mean Fluorescence Intensity, MFI) on $CD15^{+}CD66b^{high}$ **(C)** and $CD15^{int}CD66b^{int}$ **(D)** neutrophils isolated from blood and gut tissue of IBD and CRC patients and healthy controls. Data represented as mean± SD of healthy (n=3), IBD (n=6) and CRC (n=3) patients in blood, and healthy (n=3), IBD (n=9) and CRC (n=2) patients in gut tissue.

Analysis of CD301 showed a similar pattern as for CD163. CD301 was more expressed by tissue-derived neutrophils (Fig.26A,B), especially by the $CD15^{+}CD66b^{high}$ subpopulation. Only CRC-derived $CD15^{int}CD66b^{int}$ neutrophils seemed to express CD301 in the gut (Fig.26A,B).

Also expression levels reflected this trend, with tissue derived neutrophils expressing higher levels of CD301, but no major differences were observed among the two populations or among the patients type (Fig.26C,D).

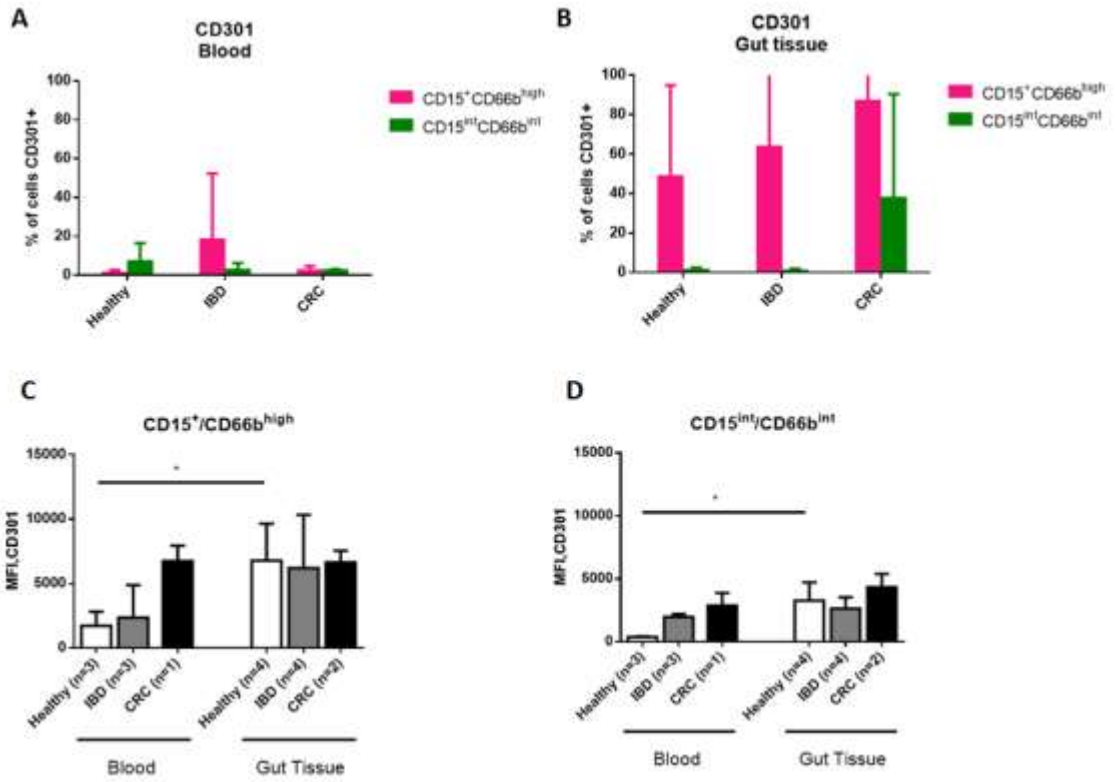

Fig.26. CD301 is differentially expressed on circulating and tissue-resident neutrophil subsets. Frequency of $CD301^{+}$ neutrophil subsets in blood **(A)** and gut tissue **(B)** of IBD and CRC patients and healthy controls. Expression levels of CD301 (referred as Mean Fluorescence Intensity, MFI) on $CD15^{+}CD66b^{high}$ **(C)** and $CD15^{int}CD66b^{int}$ **(D)** neutrophils isolated from blood and gut tissue of IBD and CRC patients and healthy controls. Data represented as mean± SD of healthy (n=3), IBD (n=7) and CRC (n=3) patients in blood, and healthy (n=4), IBD (n=9) and CRC (n=2) patients in gut tissue.

Analysis of CD206 was matching the results of CD163 and CD301. CD206 was more expressed by tissue-derived neutrophils (Fig.27A,B), especially by the $CD15^{+}CD66b^{high}$ subpopulation and, again, only CRC-derived $CD15^{int}CD66b^{int}$ neutrophils seemed to express CD206 in the gut (Fig.27A,B).

Expression levels confirmed that tissue derived neutrophils expressed higher levels of CD206, and only IBD-derived circulating neutrophils expressed high levels of CD206 while no major differences were observed among the two populations or among the patients type in the gut (Fig.27C,D).

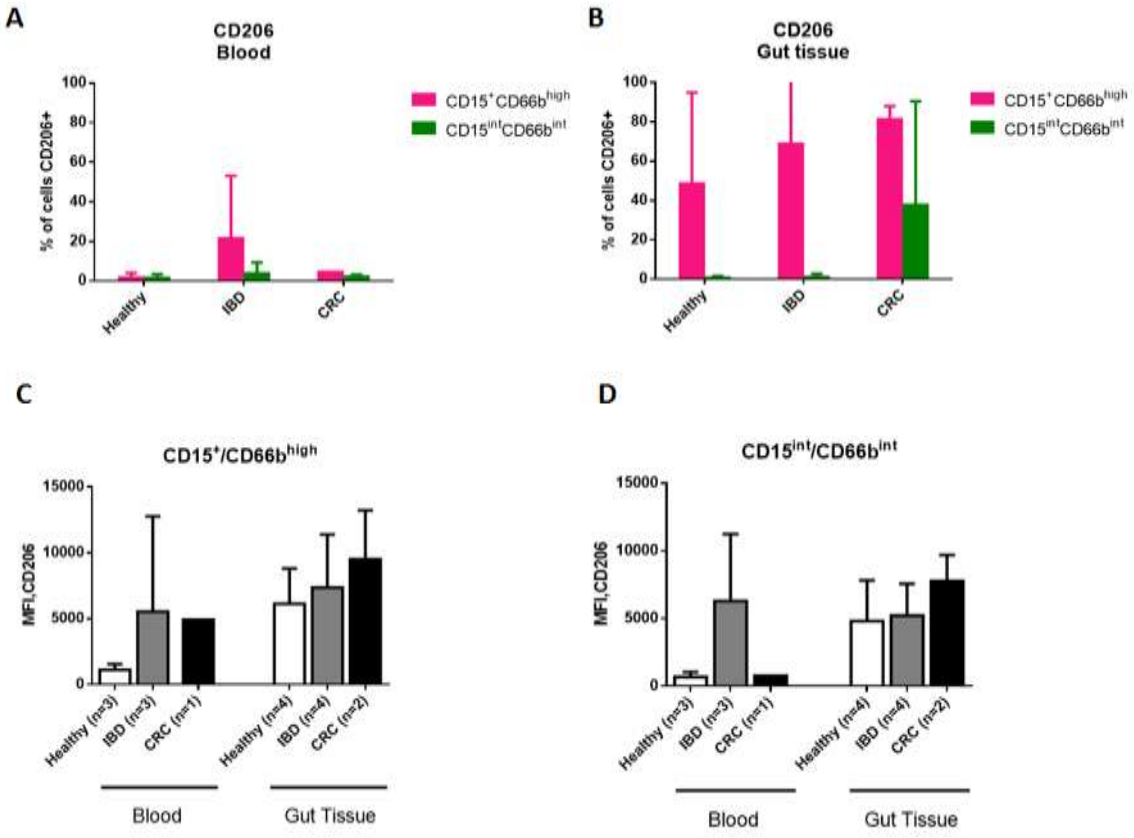

Fig.27. CD206 is differentially expressed on circulating and tissue-resident neutrophil subsets. Frequency of $CD301^{+}$ neutrophil subsets in blood **(A)** and gut tissue **(B)** of IBD and CRC patients and healthy controls. Expression levels of CD301 (referred as Mean Fluorescence Intensity, MFI) on $CD15^{+}CD66b^{high}$ **(C)** and $CD15^{int}CD66b^{int}$ **(D)** neutrophils isolated from blood and gut tissue of IBD and CRC patients and healthy controls. Data represented as mean± SD of healthy (n=3), IBD (n=3) and CRC (n=1) patients in blood, and healthy (n=4), IBD (n=4) and CRC (n=2) patients in gut tissue.

Finally, also the analysis of CD200R perfectly paralleled that of the previous N2 markers. CD200R was more expressed by tissue-derived neutrophils (Fig.28A,B), especially by the $CD15^{+}CD66b^{high}$ subpopulation (Fig. 28A,B).

Expression levels confirmed that tissue derived neutrophils expressed slightly higher levels of CD200R in the gut, with CRC-derived tissue neutrophils expressing the highest levels (Fig.28C,D).

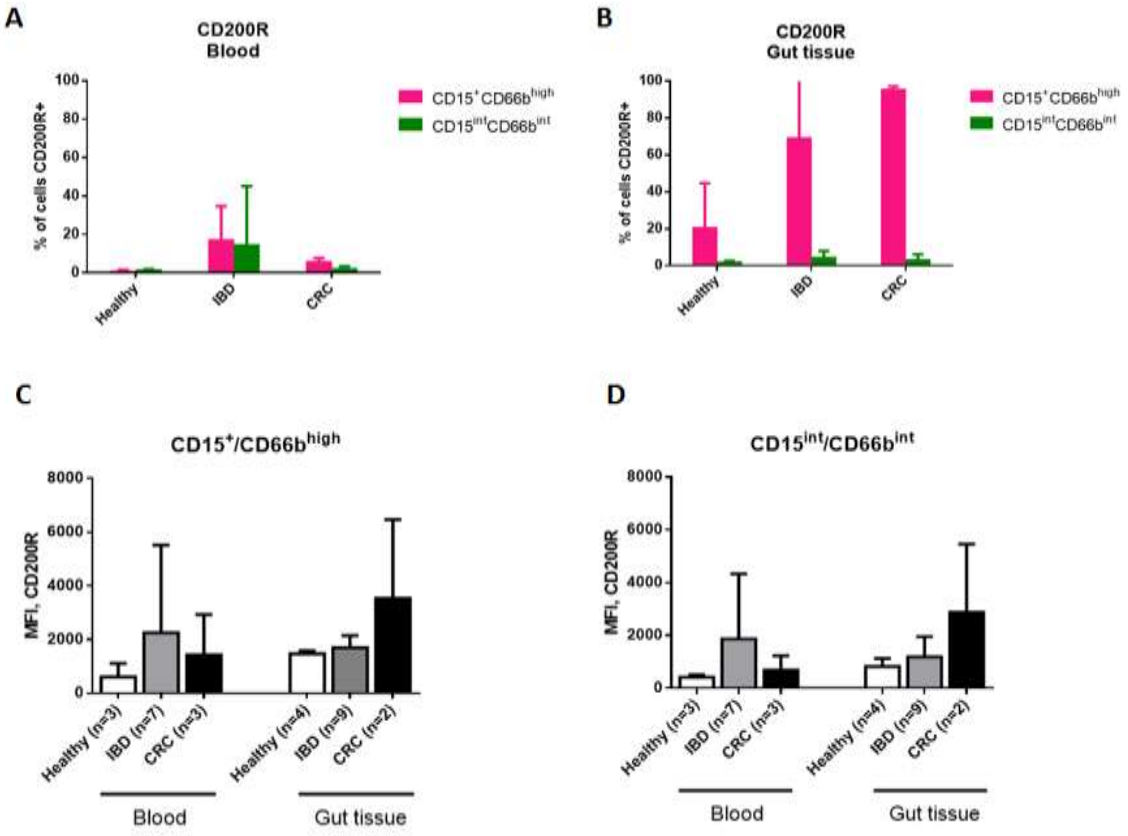

Fig.28. CD200R is differentially expressed on circulating and tissue-resident neutrophil subsets. Frequency of $CD301^{+}$ neutrophil subsets in blood **(A)** and gut tissue **(B)** of IBD and CRC patients and healthy controls. Expression levels of CD301 (referred as Mean Fluorescence Intensity, MFI) on $CD15^{+}CD66b^{high}$ (C) and $CD15^{int}CD66b^{int}$ **(D)** neutrophils isolated from blood and gut tissue of IBD and CRC patients and healthy controls. Data represented as mean± SD of healthy (n=3), IBD (n=7) and CRC (n=3) patients in blood, and healthy (n=4), IBD (n=9) and CRC (n=2) patients in gut tissue.

Finally, as we did for N1-associated markers co-expression, we evaluated the co-expression of N2-associated molecules. We found that the $CD15^{+}/CD66b^{high}$ population was mostly associated with an N2 phenotype in the gut (Fig.29), both when we compared the co-expression of CD206 and CD301 (Fig.29A,B) and of CD163 and CD200R (Fig.29C,D). Co-expression of CD163 and CD200R was the only combination of N2 markers that distinguished $CD15^{+/}CD66b^{high}$ isolated from healthy individuals from those isolated from IBD or CRC patients (Fig 29D).

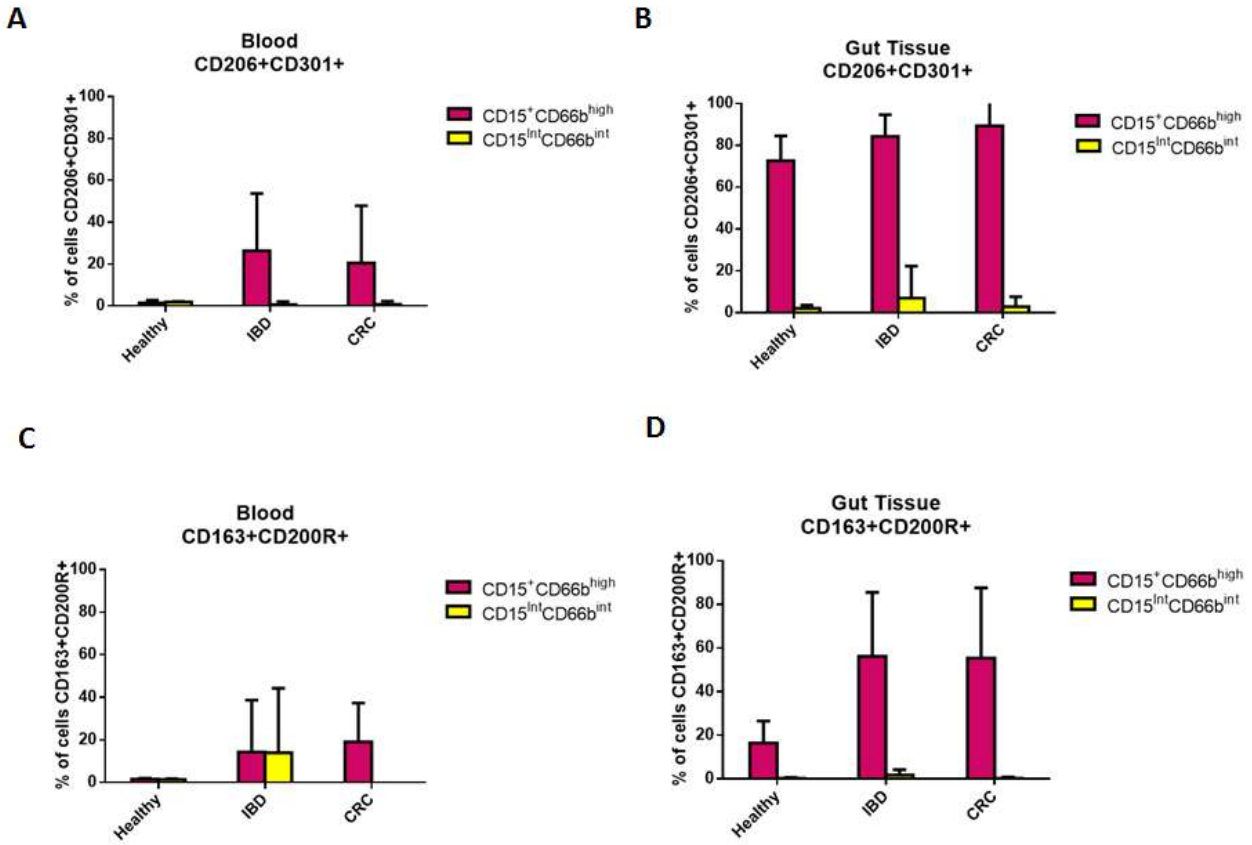

Fig.29. Human PMN subsets expressing a N2 phenotype. Histograms showing the percentage of $CD15^{+}CD66b^{high}$ and $CD15^{int}CD66b^{int}$ neutrophils with N2 phenotype in blood and gut tissue of patients and controls.

Table 8 summarizes some of the differences concerning the expression of surface markers on $CD15^{+}CD66b^{high}$ and $CD15^{int}CD66b^{int}$ PMN subsets in blood and gut tissue of patients and healthy individuals.

	$CD15^{+}CD66b^{high}$			**$CD15^{+}CD66b^{high}$**			**$CD15^{int}CD66b^{int}$**			**$CD15^{int}CD66b^{int}$**		
	Blood			**Gut Tissue**			**Blood**			**Gut tissue**		
	Healthy	**IBD**	**CRC**	**Healthy**	**IBD**	**CRC**	**Healthy**	**IBD**	**CRC**	**Healthy**	**IBD**	**CRC**
HLA-DR	+	+	+	++	+	++	+	+	+	++	++	++
CD1d	+++	+++	++	+++	++	++++	+++	++	++	+++	++	+++
CD86	+++	+++	+++	++	++	++	++	++	++	++	++	+
PD-L1	++	++	++	+++	+	++	+	+	++	++	+	+
CD54	++	++	++	+	++	++	+	++	+	+	++	++
CXCR4	++	++	++	+++	++	++	++	+	+	+++	++	++
CD62L	+	+	+	+	+	+	+	+	+	+	+	+
CD163	++	+++	++	+	+++	+	+	+++	+	+	++	+
CD301	+	+	++	++	++	++	+	+	+	++	+	++
CD206	+	++	++	++	++	++	+	++	+	++	++	++
CD200R	+	+	+	+	+	+	+	+	+	+	+	++

+=MFI,<3000; ++=MFI,>3001-10000; +++=MFI,>10001-40000; ++++=MFI,>40001

Table 8: Expression levels (calculated as MFI) of surface markers on human neutrophil subsets isolated from blood and gut tissue of IBD and CRC patients, and healthy controls.

4.2.5.3 N1 and N2 phenotype and clinical correlation

Since we did not observe major differences concerning N1/N2 polarization in the patients analysed, we asked whether different treatments might influence the phenotypical polarization of neutrophils.

4.2.5.4 Correlation between N1 phenotype and therapies

To evaluate if the use of specific immunosuppressive drugs had an impact on polarization towards the N1 phenotype of circulating and tissue-derived neutrophils, we compared the frequency of $CD15^{+}CD66b^{high}$ and $CD15^{int}CD66b^{int}$ subsets in blood and tissue of IBD and CRC patients undergoing or not immunosuppressive therapy (Fig.30). Although we had the possibility to perform this correlation on a limited number of patients, we observed a decrease of $CD15^{+}CD66b^{high}$ cells with N1 phenotype in both IBD and CRC patients under immunosuppressive therapy.

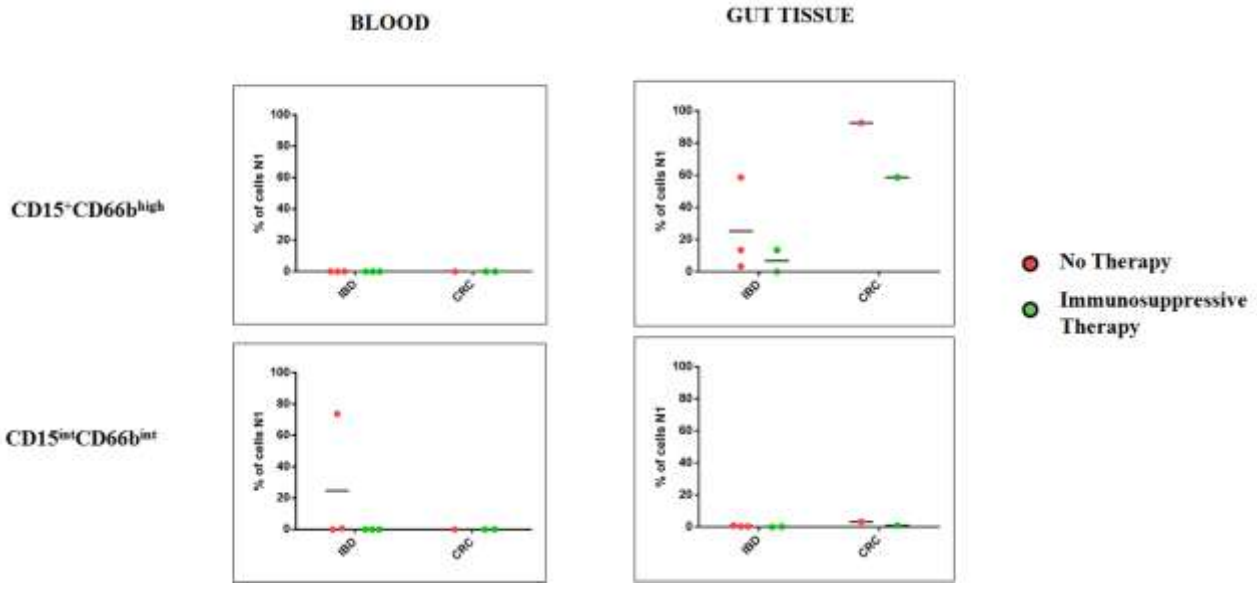

Fig.30. Immunosuppressive therapy decreases the frequency of tissue resident N1 $CD15^{+}CD66b^{high}$. Scatter dot plots showed frequencies of PMN subsets with N1 phenotype in blood and gut tissue of IBD and CRC patients with (green) or without (red) immunosuppressive therapy.

Similarly to evaluate if the use of specific immunosuppressive drugs had an impact on polarization towards the N2 phenotype of circulating and tissue-derived neutrophils, we compared the frequency of $CD15^{+}CD66b^{high}$ and $CD15^{int}CD66b^{int}$ subsets in blood and tissue of IBD and CRC patients undergoing or not immunosuppressive therapy, as reported before in Fig 30. As showed in Figure 31, the frequency of both circulating and infiltrating neutrophil subsets with N2 phenotype decreased upon immunosuppressive treatment, especially in IBD patients.

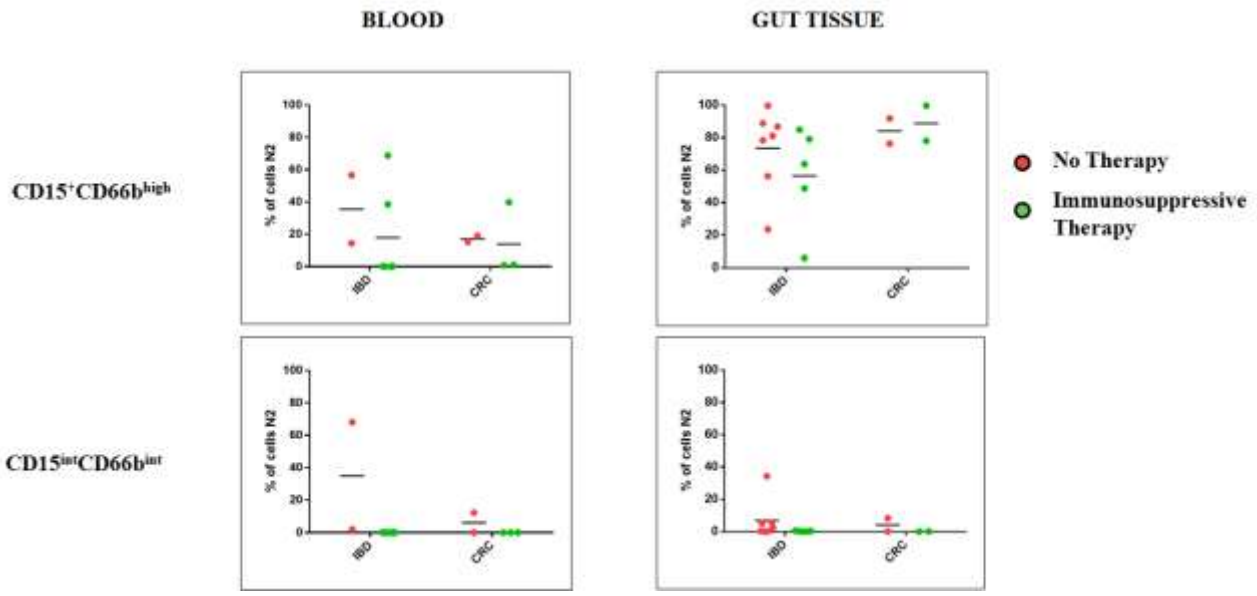

Fig.31. Immunosuppressive therapy decreases the frequency of N2 neutrophil subsets. Scatter dot plots showed frequencies of PMN subsets with N2 phenotype in blood and gut tissue of IBD and CRC patients with (green) or without (red) immunosuppressive therapy.

4.2.5.5 Correlation between N1/N2 polarisation in UC and CD patients and therapies

Firstly we analysed the frequency of polarised N1/N2 subsets among $CD15^{+}CD66b^{high}$ and $CD15^{int}CD66b^{int}$ neutrophils in blood and intestinal tissues of CD and UC patients (Fig.32).

As shown in Figure 32, both $CD15^{+}/CD66b^{high}$ neutrophils increased in the gut of CD patients showing a stronger skew towards a N2 phenotype (70% N2 vs 20% N1). In UC patients, even $CD15^{+}CD66b^{high}$ with N2 phenotype were strongly accumulating in the intestinal mucosa of the patients rather than in blood. The frequency of circulating and tissue $CD15^{int}CD66b^{int}$ cells was generally very low in both UC and CD patients (Fig 32B,C).

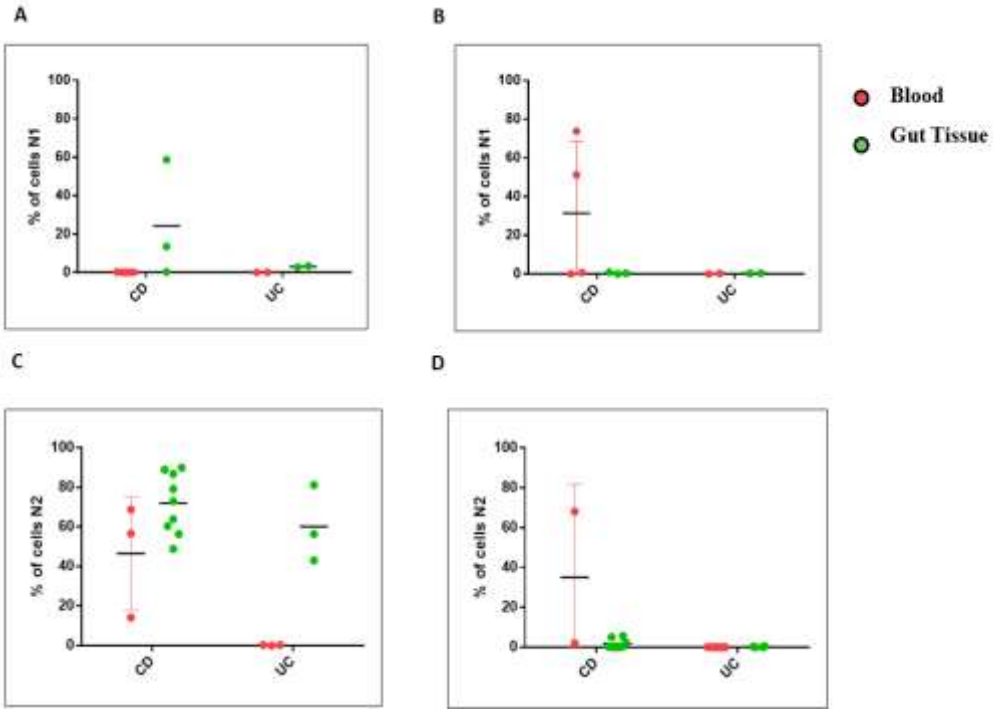

Fig.32. Circulating and tissue-derived $CD15^{+}CD66b^{high}$ neutrophils expressing N2 phenotype are increased in both UC and CD patients. Scatter dot plot showed the percentage of $CD15^{+}CD66b^{high}$ **(A-C)** and $CD15^{int}CD66b^{int}$ **(B-D)** cells with N1 **(A-B)** and N2 **(C-D)** phenotype in blood and gut tissue of CD and UC patients.

To evaluate if immunosuppressive therapy had an impact on the polarization of neutrophils subsets of CD and UC patients, we compared the frequency of $CD15^{+}CD66b^{high}$ and $CD15^{int}CD66b^{int}$ subsets in blood and tissue of these patients with or without immunosuppressive therapy ongoing. As reported in Fig.33, the frequency of both circulating and tissue derived N2-skewed $CD15^{+}CD66b^{high}$ neutrophils isolated from CD and UC patients under immunosuppressive therapy was strongly decreased.

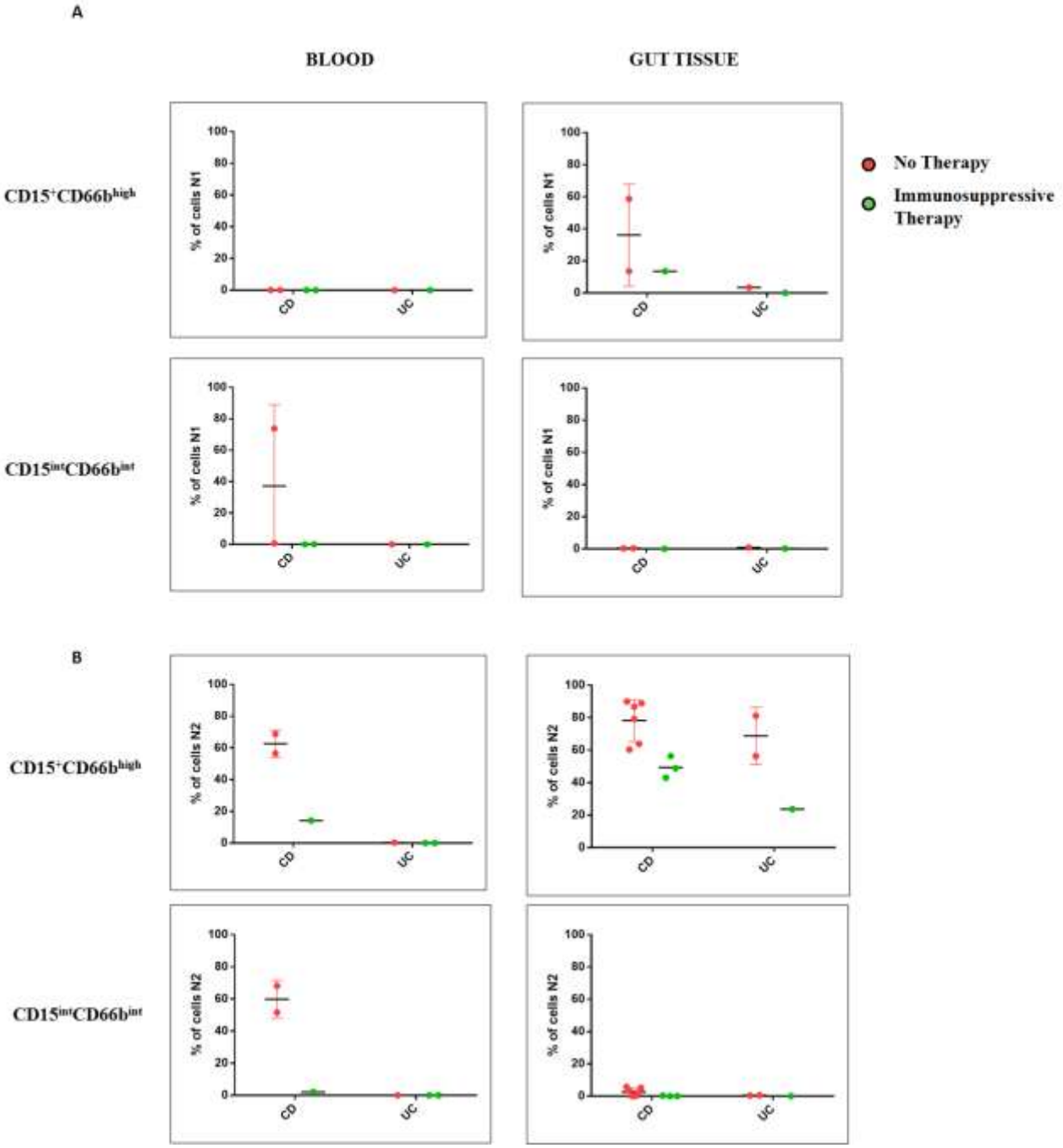

Fig.33. Immunosuppressive therapy decreases the frequency of N2 polarized neutrophils. Scatter dot plots showed frequencies of $CD15^{+}CD66b^{high}$ with N1 **(A)** and N2 **(B)** phenotype in blood and gut tissue of CD and UC patients with (green) or without (red) immunosuppressive therapy.

4.3 Functional characterization of $CD15^{+}/CD66b^{high}$ and $CD15^{int}/CD66b^{int}$ neutrophil subpopulations

Immunomodulatory function of neutrophil cells is strongly associated to the secretion of a different array of cytokines, that, similarly to human macrophages, are capable to suppress or enhance immune responses (A. Mantovani et al., 2002; S. Gordon et al., 2005; A. Mantovani et al., 2012).

In order to functionally characterise the human neutrophils subsets isolated from healthy individuals and IBD and CRC patients in blood and in the gut, we decided to perform different types of analyses. Firstly, we evaluated the cytokine profile of neutrophils (Fig.34,35) and then we evaluated their functional response upon bacterial stimulation (Fig.36).

4.3.1 Ex vivo cytokine secretion by neutrophil subsets

Freshly isolated human neutrophils were evaluated for their ability to secrete IFN-γ, a N1- derived pro-inflammatory cytokine, and IL-10, the prototypic N2 anti-inflammatory cytokine (Saraiva et al., 2010) which is implicated, among other functions, in the maintenance of gut homeostasis (Balk SP et al.,1994).
Figure 34A showed representative dot plots of IL-10 and IFN- γ released by human PMN subsets in blood and intestinal tissue of healthy individuals, IBD patients and CRC patients.
Surprisingly, the analyses did not show a clear skewed production of cytokines by the two populations of neutrophils, neither concerning the type of patients (CRC/IBD vs healthy) nor regarding the tissue localization (blood vs gut) (Fig. 34, 35).

Both $CD15^{+}/CD66b^{high}$ and $CD15^{int}/CD66b^{int}$ human neutrophil subsets were able to secrete higher levels of IL-10 in the gut as compared to blood. Although not significant, there was a tendency in CRC patients to secrete more IL-10 as compared to IBD patients and controls (Fig. 35A,B).

On the contrary, $CD15^{+}/CD66b^{high}$ neutrophils secreted lower levels of IFN-γ in the gut of IBD patients and healthy controls as compared with blood (Fig. 35C,D).

Interestingly, neutrophils isolated from CRC patients were able to secrete IFN-γ, suggesting a potential contribution to anti-tumor immune functions in the gut.

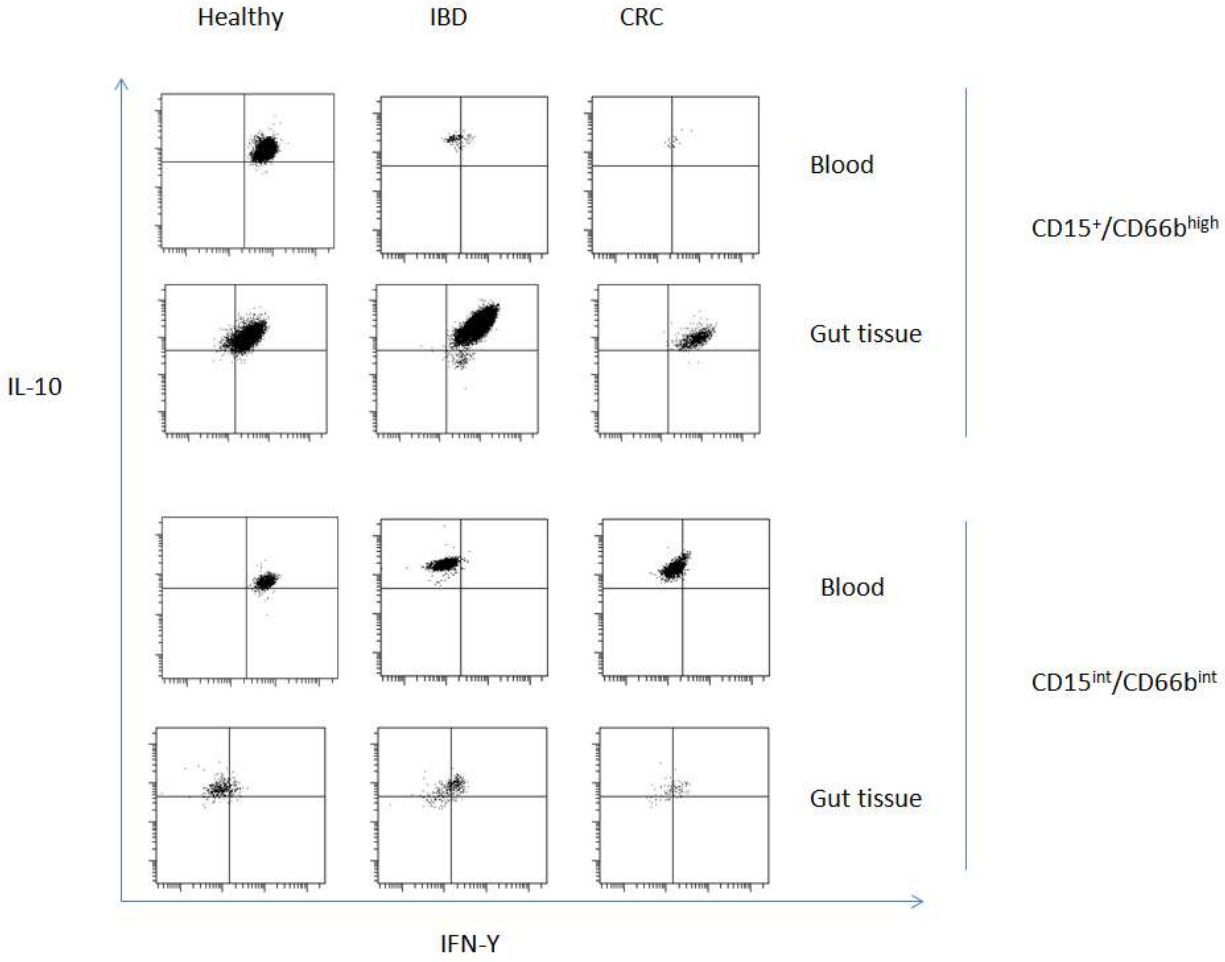

Fig.34. IL-10 and IFN-Y production by human PMN subsets in blood and gut tissue of patients and controls. Representative dot plots showing IFN-γ and IL-10 production by $CD15^{+}CD66b^{high}$ and $CD15^{int}CD66b^{high}$ in blood and gut tissue of patients and healthy individuals.

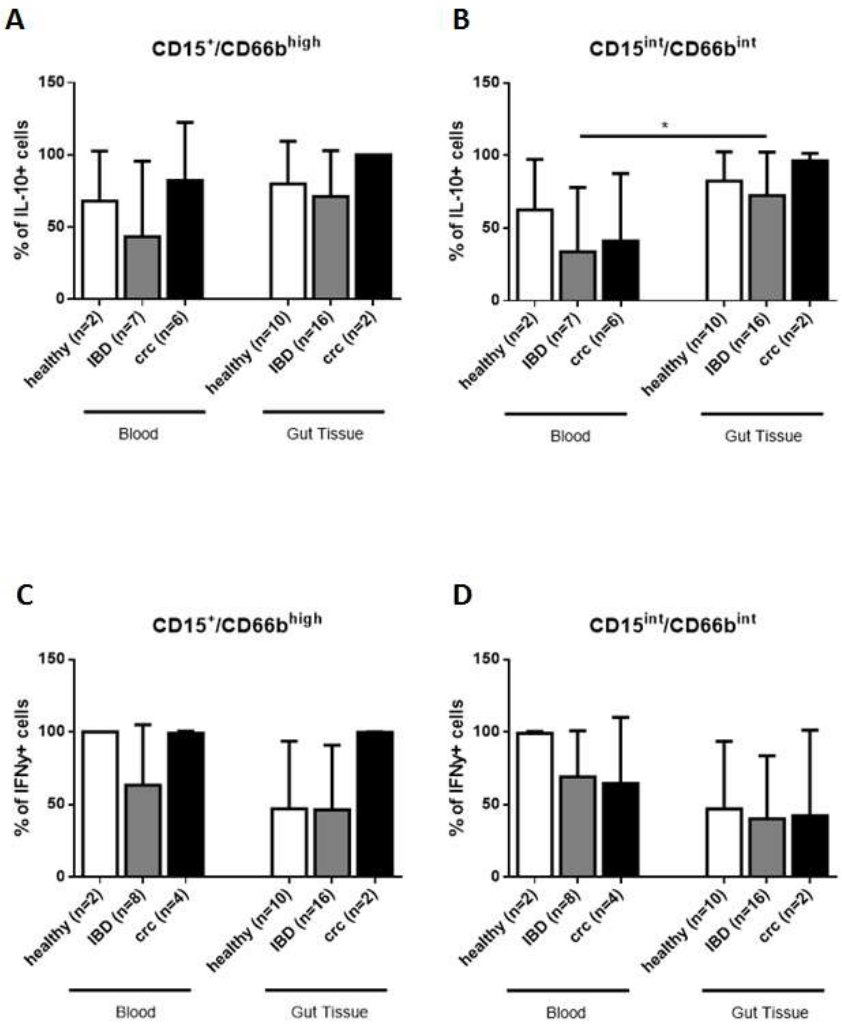

Fig.35. IL-10 and IFN-Y production by human PMN subsets in blood and gut tissue of patients and controls. Histograms showing IL-10 (A-B) and IFN- γ (C-D) secretion by PMN subsets in blood and gut tissue of patients and healthy individuals.

4.3.2 Cytokine secretion upon TLR in vitro stimulation

To investigate which were the stimuli driving the observed cytokine production by neutrophils in vivo, we stimulated freshly isolated human PMN with different bacterial stimuli. To note, we encountered several methodological difficulties to perform in vitro experiments with *ex-vivo* isolated human neutrophils, mainly due to their low vitality, additionally compromised after the long protocol of lamina propria cell isolation from intestinal tissues. Moreover, cytofluorimetric separation of neutrophils was further reducing their functional capabilities (data not shown).

We stimulated in vitro neutrophils with different TLR ligands, for 24h. Since neutrophils express TLR4, TLR3 and TLR2 (Underhill DM. et al., 2002), we stimulate them with LPS (TLR4L), Poly I:C (TLR3L) and Pam3Cys (TLR2L) (Fig. 36A) . As a read out, we analyzed a panel of pro- and anti-inflammatory cytokines such as IFN-γ, IL12p70, IL23 and IL-10.After in vitro stimulation, only blood-derived human PMN were able to secrete cytokines, while tissue- derived neutrophils were completely unable to do so. In addition, the only cytokine that we were able to detect in the culture supernatants was IL-10 after LPS -driven TLR4 triggering (Fig. 36B).

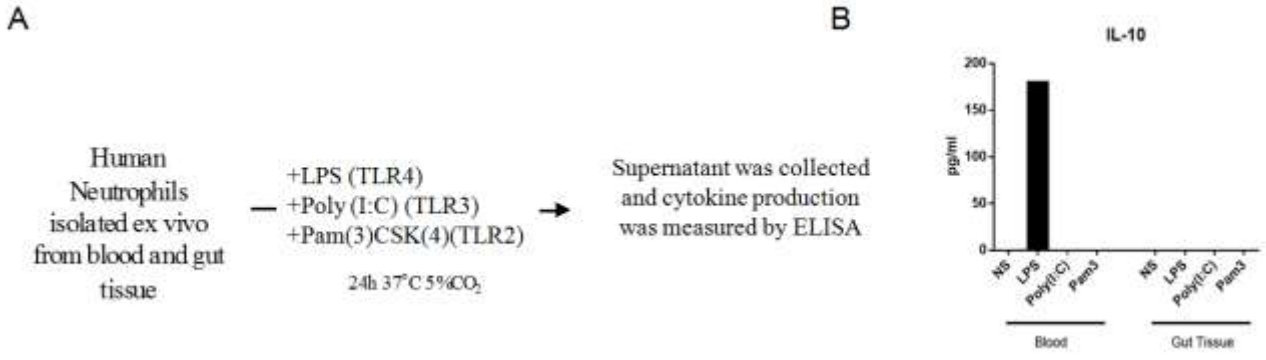

Fig. 36. TLR4 stimulation induces IL-10 production by circulating PMN. Human neutrophils were stimulated with different TLR ligands and supernatants were tested by ELISA **(A)**. Histograms represent IL-10 released by human neutrophils after bacteria stimuli **(B)**.

4.3.3 Cytokine secretion upon total luminal/mucosal bacteria in vitro stimulation

IBD and CRC patients are characterized by gut bacterial dysbiosis (Iradj Sobhani et al., 2011; C P Tamboli et al., 2004). To evaluate if a dysbiotic microbiota could influence the observed cytokine production by PMNs, we analyzed cytokines producted by neutrophils after exposure to autologous luminal and mucus-associated bacteria (Fig.37).

When circulating human PMN isolated from patients and healthy individuals were stimulated with bacteria, we observed that neutrophils from CRC patients secreted significant amount of IL-10 but almost no IL12p70 (Fig.37A,B). Conversely, neutrophils isolated from healthy individuals and from IBD patients secreted mostly IL12p70 and almost no IL-10 (Fig.37A,B).

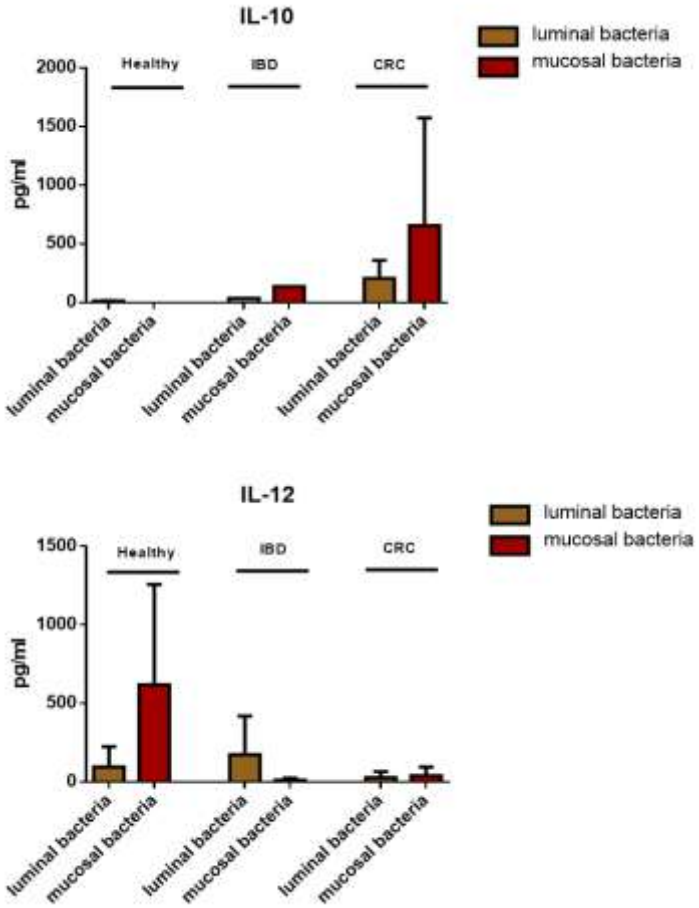

Fig. 37. Bacterial stimulation induced IL-10 and IL-12 production by human PMN of IBD, CRC and healthy individuals. Histograms represent IL-10 **(upper panel)** and IL-12 **(lower panel)** cytokine production by neutrophils stimulated with luminal (brown bar) or mucosal (red bars) bacteria, for 24 hours. Supernatant were tested by ELISA.

4.3.4 ROS production upon total luminal/mucosal bacteria in vitro stimulation

Although we observed cytokine production by neutrophils, it is well known that one of the primary functional outcomes of neutrophils activation upon bacterial stimulation is the production of Reactive Oxygen Species (ROS). To better understand the effects of intestinal bacteria on neutrophils ROS production, we stimulated in vitro blood-derived neutrophils with luminal and mucus-associated bacteria from IBD, CRC and healthy individuals. ROS production was measured by flow cytometry using an oxidant-sensitive probe DHR123 (DHR) that upon interaction with free radicals, is oxidized, resulting in the liberation of rhodamine123, a highly fluorescent molecule.

As it is shown in Figure 38, luminal and mucosa-associated bacterial stimulation induces ROS production by neutrophils isolated from healthy individuals and from IBD and CRC patients. Interestingly, neutrophils isolated from peripheral blood of IBD patients (Fig. 38B,C) seemed to react more intensely to bacterial stimulation than neutrophils isolated from CRC patients and from healthy individuals (Fig. 38B,C).

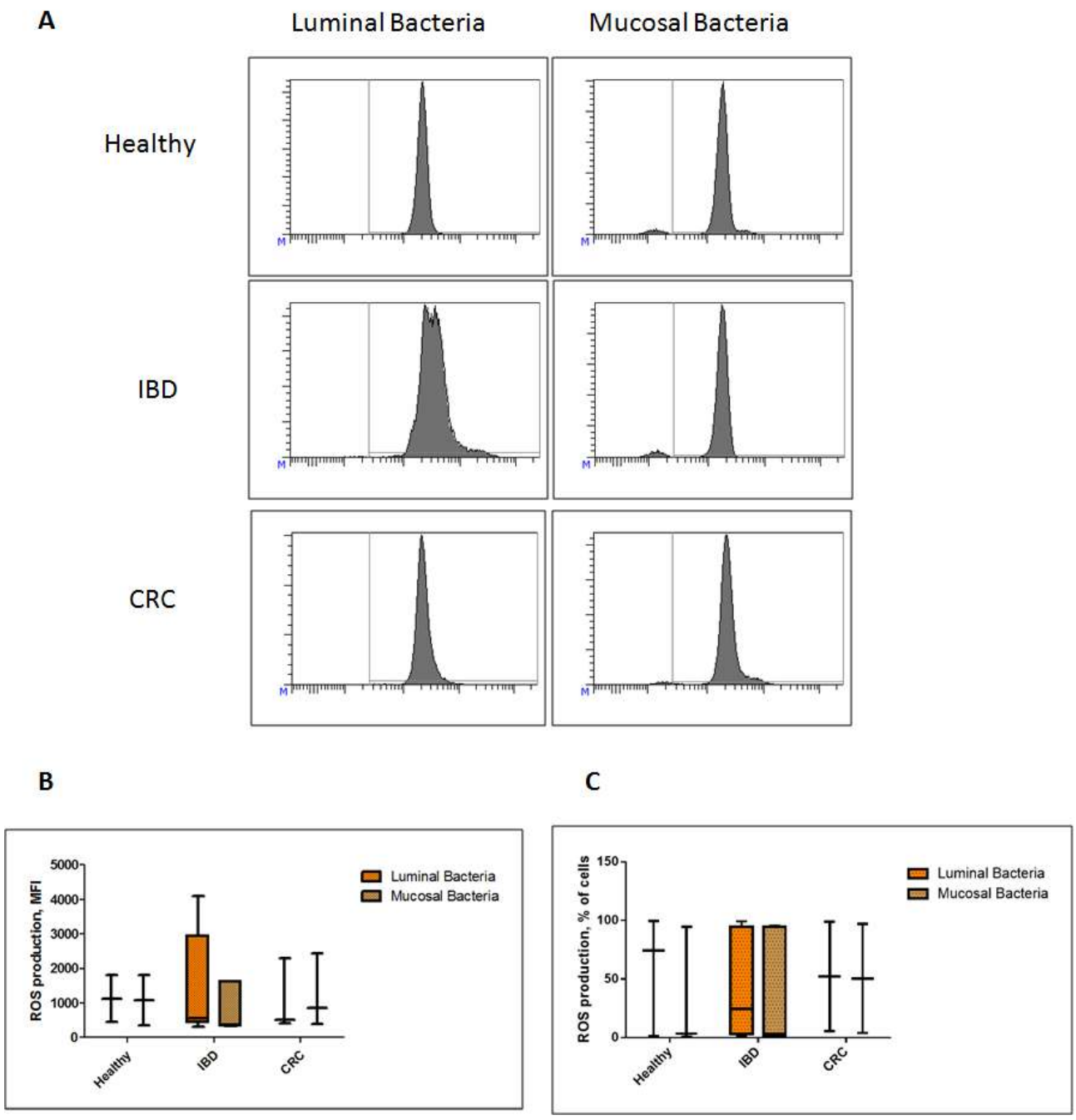

Fig. 38. Neutrophils produce ROS following bacterial stimulation. Neutrophils were stimulated with luminal and mucosal bacteria of CRC patients, IBD patients and healthy individuals. Cells were stained with DHE and ROS production was analyzed by FACS. Histograms represent ROS production **(A)**, analysis of MFI **(B)** and frequency of PMN cells **(C)** that release ROS after luminal (orange bars) and mucosal bacteria stimuli (brown bars).

DISCUSSION

Intestinal tissue of patients with active inflammatory bowel disease and colorectal cancer is heavily infiltrated by innate and adaptive immune cells, including granulocytes, macrophages and T and B lymphocytes. At present, the functional role of tissutal neutrophils in the modulation of mucosal immune responses in IBD and CRC is still largely unknown. Similarly, whether mucosal neutrophils play different roles in these two clinical conditions is still debated. In murine models of intestinal inflammation and cancer, a change in neutrophil phenotype and its possible functional consequence has already been described. However, there are limited data about the phenotype and function of neutrophil subsets in humans. In particular, the role of Tumor-associated neutrophils (TANs) in tumor development and promotion is beginning to be investigated in murine models, but it remains largely unexplored in humans.

In this thesis, we focused our attention to circulating and tissutal human neutrophils isolated from patients with IBD, CRC patients and controls. We extensively characterized them phenotipically and we evaluated if the differences observed among groups of patients might correlate with specific therapies.

Firstly, we isolated neutrophils from blood and intestinal tissue of IBD and CRC patients and from healthy individuals. While isolation of neutrophils from blood takes advantage from a well known and standardized protocol, i.e. the Dextran sedimentation followed by Ficoll-Paque density gradients, to isolate PMN from lamina propria we were forced to adapt a protocol previously described by Monteleone (Monteleone G et al., 1997). Indeed, currently few methodologies to isolate neutrophils from intestinal surgical specimens are described. The major implementation to the Monteleone protocol that we introduced was to shorten the protocol since we realized that our greatest limitation to perform subsequent analyses with freshly isolated intestinal neutrophils was their short life span and their high susceptibility to toxic agents. Neutrophils have long been considered to be a relatively homogeneous cell population. Emerging data have now confirmed that,

rather than being an end-stage uniform cell population, neutrophils can show a great level of plasticity and display distinct phenotypes and/or subsets in response to a wide range of physiological and pathological (e.g. inflammation and infection) conditions similarly to lymphocytes (Miller JFAP. 2002; Bluestone JA et al., 2009; Matthias P et al.,2005; Hirahara K et al., 2011) and monocytes (Auffray C et al., 2009). Measurement of surface receptor expression on neutrophils is often used for the evaluation of systemic inflammation in peripheral blood (Fortunati E et al., 2009). Changes in the surface expression levels of specific molecules on leucocytes is often associated with an activated phenotype and correlates with an augmented chemotaxis and transendothelial migration in vitro (Warringa RA et al., 1993; Schymeinsky J et al., 2007). Similarly, under certain pathological conditions, neutrophils are able to differentiate into different subsets, each one characterized by unique phenotype and functional profile (Scapini P et al., 2014; Sagiv JY et al.,2015).

Upon cytofluorimetric analyses of circulating and tissue-derived neutrophils, we were capable to identify two distinct subsets of human PMN differentially expressed in blood and intestinal tissue of patients with IBD, colorectal cancer and healthy individuals.

These two subsets were characterized by different expression levels of the surface molecules CD15 and CD66b. CD15 is a molecule associated to human neutrophils, and it is highly expressed on tumor-associated neutrophils (Eruslanov EB et al., 2014).CD66b, instead, was shown to be up-regulated on intratumoral neutrophils in patients with resectable non small cell lung cancer (Ilie M. et al., 2012).

Recent studies though, showed that CD15 was expressed also in granulocytic human myeloid-derived suppressor cells (G-MDSC) that are present in low numbers in the blood of healthy donors (Paul R et al., 2016) but are abundantly increased in the blood of patients with specific tumor types (Paul R et al., 2016). In mice, these cells are defined by expression of $CD11b^{+}Gr\text{-}1^{high}Ly\text{-}6G^{+}Ly\text{-}6C^{low}$ cells (Youn JI et al., 2008). In humans, neutrophils lack a marker similar to Ly6G and various studies have used different criteria for recognizing different phenotypes of MDSCs (Solito S. et

al., 2014). MDSC are morphologically and phenotipically similar to neutrophils, which raises the possibility that neutrophils in an activated state are in fact G-MDSC. Throughout the thesis work we were perfectly aware that cells with a similar phenotype (and possibly similar in vivo function) to that we described for our neutrophils were either called "MDSC" or "neutrophils" by different investigators both in cancer patients and in tumor-bearing mice (Youn JI et al., 2008; Ko JS et al., 2009; Rodriguez PC et al., 2009; Fridlender ZG et al., 2009; Schmielau J et al., 2001). This point is not simply an issue of semantics or nomenclature, but it is important to understand the biology of neutrophils and their role in pathological processes.

Keeping this issue in mind, in addition to the co-expression of CD15 and CD66b, we try to further characterise neutrophils by the additional expression of molecules known to be present on their surface.

Our results clearly demonstrated the presence of two subsets, $CD15^{+}CD66b^{high}$ and $CD15^{int}CD66b^{int}$, differentially expressed in the blood and in the lamina propria of patients with IBD, CRC and in healthy individuals. The $CD15^{+}CD66b^{high}$ population was enriched in intestinal tissues, while $CD15^{int}/CD66b^{int}$ cells represented the dominant circulating neutrophil population, especially in IBD and CRC patients.

The $CD15^{+}CD66b^{high}$ population was characterized by a higher expression of HLADR and CXCR4 inside CRC tumors but not in the inflamed mucosa of IBD patients. The vast majority of $CD15^{+}CD66b^{high}$ cells co-expressed HLA-DR and CD86 in the intestinal tissue as compared with the circulating population. While no differences were observed concerning the expression of the inhibitory molecule PD-L1 among IBD and CRC patients, in healthy subjects a higher proportion of tissue-associated $CD15^{+}CD66b^{high}$ cells expressed PD-L1. On the contrary, CD54, a molecule necessary for neutrophil transmigration through the endothelial monolayer, was expressed especially in IBD patients rather than in healthy individuals.

Also the $CD15^{int}CD66b^{int}$ population expressed higher levels of HLA-DR in the lamina propria rather than in blood. These cells also expressed higher levels of CD1d

inside the tumors as compared with blood, and CXCR4 was more expressed by tissue-derived $CD15^{int}CD66b^{int}$ neutrophils.

Similarly to $CD15^{+}CD66^{high}$ neutrophils, also $CD15^{int}CD66b^{int}$ showed an increased expression of CD54 in IBD patients as compared to the other subjects, especially in the blood. To note, a higher number of these circulating cells expressed also the L-selectin, that facilitate the infiltration of neutrophils into the site of inflammation, and it was mostly expressed by cells isolated from IBD and CRC patients.

The increased levels of antigen presenting molecules, of co-stimulatory molecules and of molecules involved in trafficking, suggest a possible role of these cells in antigen presentation in the gut. In a possible scenario, circulating neutrophils are recruited in the inflamed mucosa (of both IBD and CRC patients) by an alteration in the intestinal microflora, which has been clearly demonstrated for IBD and CRC patients (Liu Z et al., 2013). In the gut, then, these neutrophils might participate in the activation of other immune cells.

Our results show that, especially inside CRC tumors, there is an increased frequency of $CD15^{+}CD66b^{high}$ cells co-expressing HLA-DR and CD86, both involved in the presentation of antigens to CD4+T cells, but also of CD1d, a molecule necessary for lipid antigen presentation to iNKT cells. The role of iNKT cells to participate in antitumor responses has been documented in multiple human and animal cancer studies (Berzofsky JA et al., 2009, Cerundolo V et al., 2009). Several tumor types have been shown to express CD1d, and tumor expression of CD1d has been directly correlated with the ability of iNKT cells to induce direct tumor cytolysis in vitro and promote iNKT-mediated tumor immunity in vivo (Haraguchi K et al., 2006; Renukaradhya GJ et al., 2008). Contradictory data on the expression of CD1d in IBD patients have been reported. Page et al. reported that the expression of CD1d is higher in the affected ileum and cecum of IBD patients (Page MJ et al., 2000). However, a more recent report suggested that, in contrast to surface epithelium in the normal colon, epithelial cells derived from IBD patients do not express CD1d (Perera L et al., 2007). Neither study addressed CD1d expression on other cell types including

neutrophils, which could contribute to the activation of iNKT cells and the modulation of intestinal immune responses. Our results indicate that CD1d expression was very low on the surface of circulating and tissue-associated neutrophils isolated from IBD patients. On the contrary, HLA-DR was highly expressed on the surface of tissue-derived neutrophils isolated from the inflamed mucosa of IBD patients. As for CD1d, HLA-DR expressed by neutrophils may present antigens to CD4+T cells, thus are able to contributing to sustain the large amounts of T helper-derived pro-inflammatory cytokines observed in the lamina propria of IBD patients. In a murine colitis model induced by the adoptive transfer of CD4+ T cells PMN isolated from the inflamed colon expressed HLA-DR and CD86 (Ostanin DV et al., 2012). In contrast with our results, these findings were not confirmed in other murine colitis models or in patients suffering from chronic intestinal diseases.

We also observed that, inside CRC tumors, PD-L1 was scarcely expressed on human PMN subsets. Multiple solid tumor types including melanoma, RCC, NSCLC, thymoma and ovarian, express PD-L1 to generate an immunosuppressive tumor microenvironment and avoid T cell cytolysis (Blank C et al., 2007; Iwai Y et al., 2002; Blank C et al., 2005). Although PD-L1 blockade produced successful results in patients with advanced melanoma and NSCLC (Brahmer JR et al., 2012; Hodi FS et al., 2010), preliminary studies on CRC patients revealed that CRC tumor cells, similarly to what we observed for CRC-derived neutrophils, manifest low expression of PD-L1, suggesting that CRC-derived cells may respond less to PD-1 or PD-L1 inhibition (Taube JM et al., 2014).

Our results also showed a downregulation of CD62L and an upregulation of CD54 and CXCR4 expression on infiltrating $CD15^{+}CD66b^{high}$ neutrophils of CRC patients. These results were consistent with recent studies showing that Tumor associated neutrophils displayed a more activated phenotype ($CD62L^{low}CD54^{high}$) (Pignatti P et al., 2005; Fortunati E et al., 2009) as compared to circulating neutrophils and with the fact that CXCR4 is overexpressed in human cancers (Uhlen,& Rhim, 2010). The

migration of leukocytes into inflamed bowel tissue is central to the pathogenesis of IBD. Under inflammatory conditions, a large number of activated immune cells infiltrate the intestinal mucosa. These immune cells, among them also neutrophils, express high levels of CD62L and CD54, which further induce intermolecular interactions of leukocytes in the blood circulation to migrate into the intestinal mucosa, and promote local inflammatory response (Baumgart DC et al., 2012; Manichanh C et al., 2012; Ordás I et al., 2012; Latella G et al., 2012;Van Assche and Rutgeerts, 2005; Bernstein et al. 1998). This is confirmed by our results, that showed increased expression levels of CD54 on both PMN subsets infiltrating inflamed mucosa of IBD patients, and an increase of CD62L specifically on tissue-derived $CD15^{int}CD66b^{int}$ neutrophils.

The patients that we had the possibility to analyse were all under different types of pharmacological treatments. We considered in our analysis the possibility that these drugs might modify i) the frequency of the neutrophils sobpopulation that we identified and ii) in general the expression of some or all the surface molecules expressed by these subpopulations.

Since the pharmacological treatments were different, we grouped the patients for immunosuppressive therapy that they receiving or not at the time of surgical intervention. Immunosuppressants include thiopurines such as 6-mercaptopurine (6-MP), azathioprine (AZA), methotrexate (MTX), and steroids and anti-TNF (infliximab or adalimumab). 6- Mercaptopurine (6-MP) and azatioprine (AZA) inhibit lymphocyte proliferation through the incorporation of active drug metabolites into cellular nucleotides, which likely results in anti-inflammatory effects and in suppression of T cell function and natural killer cell activity (Sahasranaman S et al., 2008; Regueiro MD et al., 2000). These drugs also downregulate the levels of inhibitory molecules and molecules involved in trafficking, especially CD54 (Chang CZ et al., 2010).

Our results showed that immunosuppressants reduce the number of both circulating subsets of PMN in IBD and CRC patients, but upregulate the expression levels of antigen presenting molecules and co-stimulatory molecules.

In IBD and CRC-derived tissues, immunosuppressants seemed to slightly increase the frequency of the $CD15^{+}CD66b^{high}$ subpopulation, while reducing the percentage of the intermediate subset. To note, especially in the $CD15^{+}CD66b^{high}$ population, immunosuppressants reduce the expression levels of antigen presenting molecules such as CD1d, of molecules involved in trafficking such as CD54 and CD62L in IBD and CRC patients. These data suggest that this population might be a direct target of the immunosuppressive therapy, underlying their potential contribution in the intestinal pathogenesis.

Tumor-associated macrophages (TAMs) are an important component of the inflammatory infiltrate of several tumors (Mantovani et al., 1992) and are essential mediators of the relationship between inflammation and cancer. In the tumor microenvironment macrophages can express pro- or anti-tumoral functions. This plasticity reflects macrophages peculiarity to acquire distinct phenotypes in response to distinct microenvironmental signals (Sica and Mantovani, 2012). Relatively little is known about neutrophils in human cancers and IBD and if, in different inflammatory or tumor-associated environments, also neutrophils might acquire distinct phenotypes. Recent evidences suggest that tumor infiltrating neutrophils, termed TANs, undergo polarization toward distinct phenotypes in response to certain tumoral environmental signals (Fridlender et al., 2009). To date, two subsets of TANs with distinct phenotypes and properties have been characterized (Fridlender ZG et al.,2009; Piccard H et al., 2012). The majority of TANs in the tumor microenvironment exhibit an immunosuppressive and pro-tumorigenic phenotype, a subset termed N2 similarly to the macrophage counterpart M2.

Melanoma patients also show a specialized subset of immunosuppressive neutrophils in their blood that is induced by serum amyloid A1 (SAA-1), produces the anti-inflammatory cytokine IL-10 and promotes the interaction of these neutrophils with

invariant natural killer T cells that decreases their IL-10 production while enhancing IL-12, thereby dampening their immunosuppressive properties, again resulting in the existence of two distinct neutrophil populations with defined functions (De Santo C et al., 2000). The presence of TANs have been demonstrated to be associated with poor clinical outcomes of several malignancies including clear cell renal cell carcinoma (Jensen HK et al., 2009), gastric cancer (Zhao JJ et al., 2012),colorectal cancer (Rao HL et al., 2012), and hepatocellular carcinoma (Li YW et al., 2011).

Recent studies showed that M1 macrophages, the "inflammatory" macrophage phenotype, displayed significantly higher CD86 and HLA-DR levels as compared to M2 (Buchacher T et al., 2015), while M2 macrophages expressed subset-specific markers include a scavenger receptor CD163, a galactose-type C-type lectin, CD301 (Prokop S et al., 2011; Raes G et al., 2005), and a scavenger receptor expressed by both tolerogenic macrophages and dendritic cells, CD206 (Heusinkveld M et al., 2001; Verreck FA). No unique cell-surface receptors have yet been identified for M1 macrophages (Heusinkveld M et al., 2001; Verreck FA).

We evaluated if circulating and tissue-infiltrating $CD15^{+}CD66b^{high}$ and $CD15^{int}CD66b^{int}$ neutrophils also showed N1/N2 phenotype in IBD, CRC patients and healthy individuals.

Our results showed the co-expression of CD86 and HLA-DR on $CD15^{+}CD66b^{high}$, surface markers typical of an inflammatory M1/N1 phenotype. Nevertheless, we also observed a strong increase of the frequency of $CD15^{+}CD66b^{high}$ expressing CD163, CD301, CD206 and CD200R (typical M2/N2 markers) in the inflamed mucosa of IBD patients and inside the tumor. While we were expecting a skew towards an N2 phenotype inside CRC tumors, we were surprised to find such a high proportion of neutrophils with an N2 phenotype in IBD patients.

Although unexpected, these data were also confirmed by functional data. When we characterized circulating neutrophils *ex vivo*, we found that the vast majority of cells secreted IL-10, a potent anti-inflammatory cytokine involved in N2 polarization, and $CD15^{+}CD66b^{high}$ neutrophils isolated from the inflamed mucosa of IBD patients

secreted low levels of IFN-γ. The published literature on the production of IL-10 by human neutrophils is conflicting. Despite the fact that several studies (Tsuda Y et al., 2004, Mencacci A et al., 1996; Noel Gb et al., 2011; Tosello Boari J et al., 2012) have shown that mouse neutrophils produce IL-10 in response to a variety of infections, and the data are widely accepted, only two groups of investigators were able to reproduce those results using human neutrophils (De Santo C et al., 2010; Balderramas HA et al., 2014), but other study showed that human neutrophils did not secrete IL-10 in response to various inflammation-associated molecules (Mantovani A et al., 2011).

Additional functional studies with a higher number of patients will be required to definitively support our findings.

In the gut, bacteria play different important functions, and neutrophils are immune cells deputed to sense variations in the bacterial communities, for example during pathogens infections.

To further functionally characterise ex vivo neutrophils isolated from IBD and CRC patients, and evaluate if they are endowed with distinct functional capacities, we stimulated them with different bacteria cues.

We encountered technical difficulties to perform in vitro experiments with freshly isolated neutrophils. Firstly, neutrophils are short lived and PMN isolated from the lamina propria of patients were highly stressed and died faster probably for the long time of tissue preparation. For these reasons when we used tissue-derived neutrophils to perform in vitro experiments, these cells did not respond.

We therefore decided to perform experiments with circulating neutrophils, as previously published (Hayashi F et al., 2003). As shown for monocytes, neutrophils secrete IL-10 after LPS stimulation (Nizzoli G et al., 2016).

The endogenous intestinal microflora plays a crucial role in the pathogenesis of IBD (Sartor RB et al., 2007; Shanahan F, 2012) and it has been shown that several bacteria produce toxins that disturb the cell cycle resulting in altered cell growth in cancer (Littman AJ et al., 2004; Koyi H et al., 2001; Kocazeybek B, 2003) but if

these stimuli had a direct impact on neutrophils activation is still largely unknown. We thus evaluated neutrophil responses to luminal and mucus-associated bacteria stimuli. Our results showed that under mucosal bacterial stimulation, neutrophils isolated from CRC patients secreted significant amounts of IL-10 but almost no IL12p70 while, in IBD patients, neutrophils activated by luminal bacteria, secreted IL12p70 and almost no IL-10. These data confirm that neutrophils functions are strongly influenced by exposure to intestinal bacteria and that these bacteria might directly participate in the skewing of the functional phenotype of neutrophils in IBD and CRC patients.

Finally, as known in literature, the release of cytokines was only one of different mechanism adopted by neutrophils to respond to bacterial stimulation. Neutrophil effector functions also include production and release of reactive oxygen species (ROS). Evidences showed a ROS induction by neutrophils in response to microbial signals (Lambeth JD et al., 2004). On these bases, evaluated ROS production under luminal and mucus-associated bacterial stimulation and we showed that circulating neutrophils of IBD patients seemed to react more intensely as compared with CRC patients and healthy individuals.

In conclusion, by taking advantage of different surface markers, we identified two population of neutrophils differentially expressed in the peripheral blood and in the gut tissue of patients with IBD and CRC. These two subpopulation not only expressed different surface molecules associated to antigen presentation, activation, and trafficking of neutrophils in both peripheral blood and gut mucosa, but also demonstrated phenotypical and functional differences between these patients. These populations expressed molecules associated to N2 phenotype and secreted more IL-10. Immunosuppressive therapy modulated their relative frequencies and the expression of specific surface molecules involved in their functional activities, suggesting an important role of neutrophils in the pathologies that we have studied. Finally, functional analyses revealed that these cells produce cytokines and ROS in response to the intestinal bacteria, especially mucus-associated bacteria.

Our results therefore suggested that the interaction between gut bacteria and neutrophils can contribute to the initiation and propagation of these intestinal pathologies and, in the future, the block of this interaction may be a potential therapeutic target in both IBD and CRC.

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